



IMMUNOCYTOCHEMICAL AND SYNAPTOLOGICAL CHARACTERIZATION  
OF RAT OLFACTORY BULB GLOMERULI

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Juan Carlos Bartolomei

Yale University

1994

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## ***DEDICATORIA***

*Le quiero dedicar esta tesis a mi familia. Por su continuo apoyo moral y emocional que me han brindado y su confianza que algún día lograré mis sueños.*





IMMUNOCYTOCHEMICAL AND SYNAPTOLOGICAL CHARACTERIZATION OF  
RAT OLFACTORY BULB GLOMERULI

A THESIS SUBMITTED TO THE YALE SCHOOL OF MEDICINE IN PARTIAL  
FULLFILLMENT OF THE REQUIREMENTS FOR THE  
DEGREE OF DOCTOR OF MEDICINE

BY

JUAN CARLOS BARTOLOMEI

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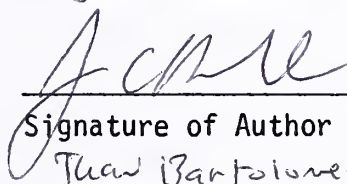
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## ABSTRACT

IMMUNOCYTOCHEMICAL AND SYNAPTOLOGICAL CHARACTERIZATION IN RAT OLFACTORY BULB GLOMERULI. Juan C. Bartolomei and Charles Greer. Sections of Neurosurgery & Neurobiology, Yale Univ. Sch. Med., New Haven, CT.

Synaptic circuits of the olfactory bulb glomerulus are not well understood. It is recognized that glomeruli include both axodendritic primary afferent synapses and dendrodendritic local circuit synapses. However, it is unknown if these are homogeneously distributed among the subpopulations of neurons contributing processes to the glomerulus. **Therefore the purpose of this thesis was to elucidate fundamental patterns of synaptic specificity and organization in the rat olfactory bulb glomerulus.** Employing immunocytochemistry, at the level of light and electron microscopy, we studied synaptic interactions involving, mitral/tufted cells, olfactory nerve terminals, and periglomerular cells immunoreactive and non-immunoreactive for GABA and tyrosine hydroxylase. Based on their immunocytochemical properties we identified four subpopulations of periglomerular: 1) immunoreactive to GABA only; 2) immunoreactive to tyrosine hydroxylase only; 3) immunoreactive to both GABA and tyrosine hydroxylase; 4) non-immunoreactive to either GABA or tyrosine hydroxylase. The majority of immunoreactive cells were immunoreactive to both GABA and tyrosine hydroxylase.

At the level of the electron microscope our observations are in accord with those previously described in the rat olfactory glomerulus. Olfactory nerve axons were characteristically electron dense containing many spherical vesicles within their axoplasm. Mitral and tufted cell processes had an electron lucent cytoplasm with several organelles such as mitochondria and smooth endoplasmic reticulum and arrays of microtubules distributed throughout the dendrites. The periglomerular cell processes had





an electron dense cytoplasm but otherwise share a number of similarities with the mitral and tufted cells such as mitochondria and smooth endoplasmic reticulum. The synaptic organization among these processes were also in accord with previous observations. Transglomerular montages revealed from a qualitative point of view several discrete levels of subglomerular organization, although a more complete quantitative synaptological analysis is required to establish significant conclusions.

The organization of tyrosine hydroxylase and GABA immunoreactive processes was assessed employing preembedding immunocytochemistry and electron microscopy. Immunoreactive and non-immunoreactive periglomerular cells were equivalent morphologically and in the type of synapses they received or made. However they differed in their patterns of synaptic distribution. The tyrosine hydroxylase immunoreactive processes received a higher density of synapses from the olfactory nerve terminals and established more symmetrical synapses onto presumed mitral/tufted cell processes than the GABA immunoreactive processes. The GABA immunoreactive processes received a higher density of synapses from presumed mitral/tufted cell processes than did the tyrosine hydroxylase immunoreactive processes. The presumed mitral/tufted cell processes received the highest density of synapses from the olfactory nerve.

The observations of a differential synaptic distribution found among the neuronal elements in the glomerulus supports the notion of an organizational heterogeneity within the glomerular structure. This subglomerular organization could imply discrete functional modules for odor processing, as seen in the functional organization of other areas of the brain. In a similar fashion, conclusions derived from the olfactory bulb glomerulus may be readily applicable to understanding the organization of other areas of the central nervous system that involve local circuits.



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20. The twentieth step is to migrate the solution.

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1. The first part of the document discusses the importance of maintaining accurate records of all transactions and activities. It emphasizes that this is crucial for ensuring transparency and accountability in the organization's operations.

2. The second part outlines the various methods and tools used to collect and analyze data. This includes both traditional manual methods and modern digital technologies, highlighting the benefits of each approach.

3. The third part focuses on the role of the data in decision-making processes. It explains how data-driven insights can help identify trends, anticipate challenges, and optimize resource allocation.

4. The fourth part addresses the security and privacy of the data. It discusses the necessary measures to protect sensitive information from unauthorized access and ensure compliance with relevant regulations.

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## INTRODUCTION

“Odor gives us the most intimate sensations, a more immediate pleasure, more independent of the mind, than the sense of sight; we get profound enjoyment from an agreeable odors at the first moment of its impression; the pleasure of sight belongs to reflections, to the desires aroused by the objects perceived, to the hopes they give birth to.” Jean-Francois Saint Lambert, *Les Saisons* (1769), 35 quoted in Mauzi, *L’Idée du bonheur*, (Corbin, 1986).

For several centuries the significance attributed to the olfactory system in our routine lives has varied. Although much is now known about the olfactory system functions, a number of important questions remain about differences in synaptic circuitry in various parts of the olfactory bulb. Three studies reported in this thesis address these questions.

The initial part of this introduction will illustrate the impact of the olfactory system in society during the late eighteenth early nineteenth century. As an example of this impact we will focus our attention on the well documented Parisian society during this time. This will be followed by dominating clinical models of olfactory pathology during the nineteenth century and proposed remedies employing the olfactory system for certain diseases. Golgi’s and Cajal’s revolutionary anatomical illustration of the olfactory system and their significance to the field of neuroscience will follow. Lastly, a detailed anatomical description of the olfactory system, including histological and neurochemical composition, and significance of the current investigation will be explained.

### A. HISTORICAL PERSPECTIVE

During the late eighteenth century the sense of smell was considered an animal sense; sniffing and smelling were defined as animal behavior. The linkage to lust, intimacy, sensuality, desire and impulsiveness further distinguished the sense of smell as those belonging to savages, unrefined men/women, and those ignorant of good manners.

## Introduction

The purpose of this study is to investigate the effect of the use of a mobile learning application on the learning outcomes of students in a mathematics course. The study is a quasi-experimental design with two groups: an experimental group and a control group. The experimental group used the mobile learning application, while the control group used traditional learning materials. The data were collected through a pre-test and a post-test. The results of the study show that the use of the mobile learning application had a significant positive effect on the learning outcomes of the experimental group compared to the control group.

The study was conducted in a mathematics course for students in the first semester of a university. The course was taught by a lecturer who had experience in teaching mathematics. The experimental group consisted of 30 students, and the control group consisted of 30 students. The data were collected through a pre-test and a post-test. The results of the study show that the use of the mobile learning application had a significant positive effect on the learning outcomes of the experimental group compared to the control group. The results also show that the use of the mobile learning application had a significant positive effect on the learning outcomes of the experimental group compared to the control group.

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For this reason, along with the sense of touch, the sense of smell was at the bottom of the hierarchy of senses. Despite the general prejudice against this sensory organ, others ascribed significant importance to it. Behavioral scientists regarded the sense of smell as the sense of self preservation. The nose was the detector of dangerous, potentially, poisonous substances. In addition the developing theories of medical infections, together with the increase recognition of hygiene, attenuated the otherwise declining interest in the sense of smell. According to Corbin (1986) “doctors since ancient times have untiringly stressed the importance of the nose as the sensory organ closest to the brain, the origin of sensation.... capable of shaking man’s inner life more profoundly than were the sense of hearing or of sight.”

The impact of odor in Parisian society during the late eighteenth century is eloquently and graphically described in Corbin’s book *The Foul and the Fragrant Odor and the French Social Imagination* (1986). The concern of contracting a horrifying disease after coming in contact with putrefying smells led to numerous myths and avoidance behavior by the eighteenth century Parisians. It was believed that the core of the earth served as a reservoir of fermenting, mysterious mixtures that with time would seep up to the surface. Therefore, any cracks, fissures, faults or abysses were cautiously approached if not totally evaded. The most feared cracks and fissures were those created by earthquakes and those found in swamps. Other anxiety provoking areas were virgin soil, and marshes with their odiferous emanation, humid soils and stagnant waters, or any proximity to water. These fears were based on the belief that humidity, in contrast to the sun, would cause the downward movement of heavy miasma to settle on the ground thus, potentially introducing a life threatening disease. The humidity on new walls and the stagnant odors accumulated on the walls of hospitals were presumed to serve as conduits for transmitting diseases such as neuralgias, acute joint or muscular pain and gangrenous disorders. After an epidemic of puerperal fever at a Lyons Hospital the mortar in the walls was removed as a mean of disinfection (Corbin, 1986).



Based on patient's personal body odors and breath, doctors attempted to diagnose and determine the extent of individual's inner decay (Althaus, 1881). This enabled doctors to identify those individuals who were on the verge of death and seclude them away. This plan of management was essential since no odor was feared more than that from a corpse, especially those emanating shortly after death. Despite being fully acquainted in working with corpses, doctors expressed a certain degree of hesitancy when dealing with a dead human being. This is exemplified by this excerpt from Charles Londe about Nicolas Chambon de Monatux, a medical student undergoing an anatomy examination using a cadaveric liver.

“struck by the putrid emanations escaping from it immediately it was opened, fainted, was carried home, and died within several hours; another, the famous Fourcroy, was affected with burning eruptions on the skin; the other two, ... languished for a long time, one not recovering.

As for Chambon, he was consumed with indignation at the dean's persistence and remained steadfastly in his place. He completed his lesson amid stewards drenching their handkerchiefs in aromatic water, and probably owed his health to that cerebral overexcitement which brought on a copious sweating during the night after a few outbreaks of fever (Corbin, 1986).”

The growing civic preoccupation and public health threat inspired several scientists to study in full detail the science of smell, better known as ophresiology. New experiments analyzed the thresholds of noxious smells, particularly in areas close to cemeteries, and established spatial boundaries for cities in order to prevent the spread of presumably stench-borne diseases.

In 1821 one of the leading ophresiologyists, Hippolyte Cloquet produced history's first reference work on the problem of olfaction in his book, *Osphrésiologie, ou Traité des odeurs, du sens et des organes de l'olfaction, avec l'histoire détaillée des maladies du*





*nez et des fosses nasales et des operations qui leur conviennent.* In his book Cloquet presents a very exhaustive and complete explanation on the nature, effects, physiology, gross anatomy and folk observations on the sense of smell. He described the functional linkage between the sense of olfaction and the digestive system, an observation that was not shared by other sensory systems. In addition, he described a physiological and psychological linkage between the sense of olfaction and the reproductive system in animals and humans. For this reason he ascribed to the sense of olfaction the responsibility for survival of the species.

During this time Cloquet's work heightened the interest in olfaction to a new level. As mentioned before, there was an increasing awareness of hygiene, but in addition a new social stratification was developing based on the stench of individuals. The French aristocracy had available for them perfumes and other colognes to mask the body's odoriferous emanations thus allowing them to isolate themselves from the proletariat. According to Corbin, the sharp difference in smell between the aristocracy and the peasants led to several civic revolts where the peasants demonstrated quite graphically and explicitly their attitudes against the aristocracy (Corbin, 1986).

In the late nineteenth century, Pasteur's revolutionary discovery of germ transmission drastically changed society's attitude toward the sense of smell. Once it was proven that diseases were caused by infectious germs, the scientific community no longer associated unpleasant odors with the threat of death or ailment. The fear of cemeteries was abolished and in 1879 a commission concluded that these places were harmless to society (Corbin, 1986). The civic attitude towards hygiene was now shifted to an understanding on the pathogenesis of diseases caused by infectious organisms. At the same time the interest in clinical diagnosis based on the smell of an individual declined and the intrigue of the sense of olfaction was focused on its relationship with other clinical pathologies and manifestations.

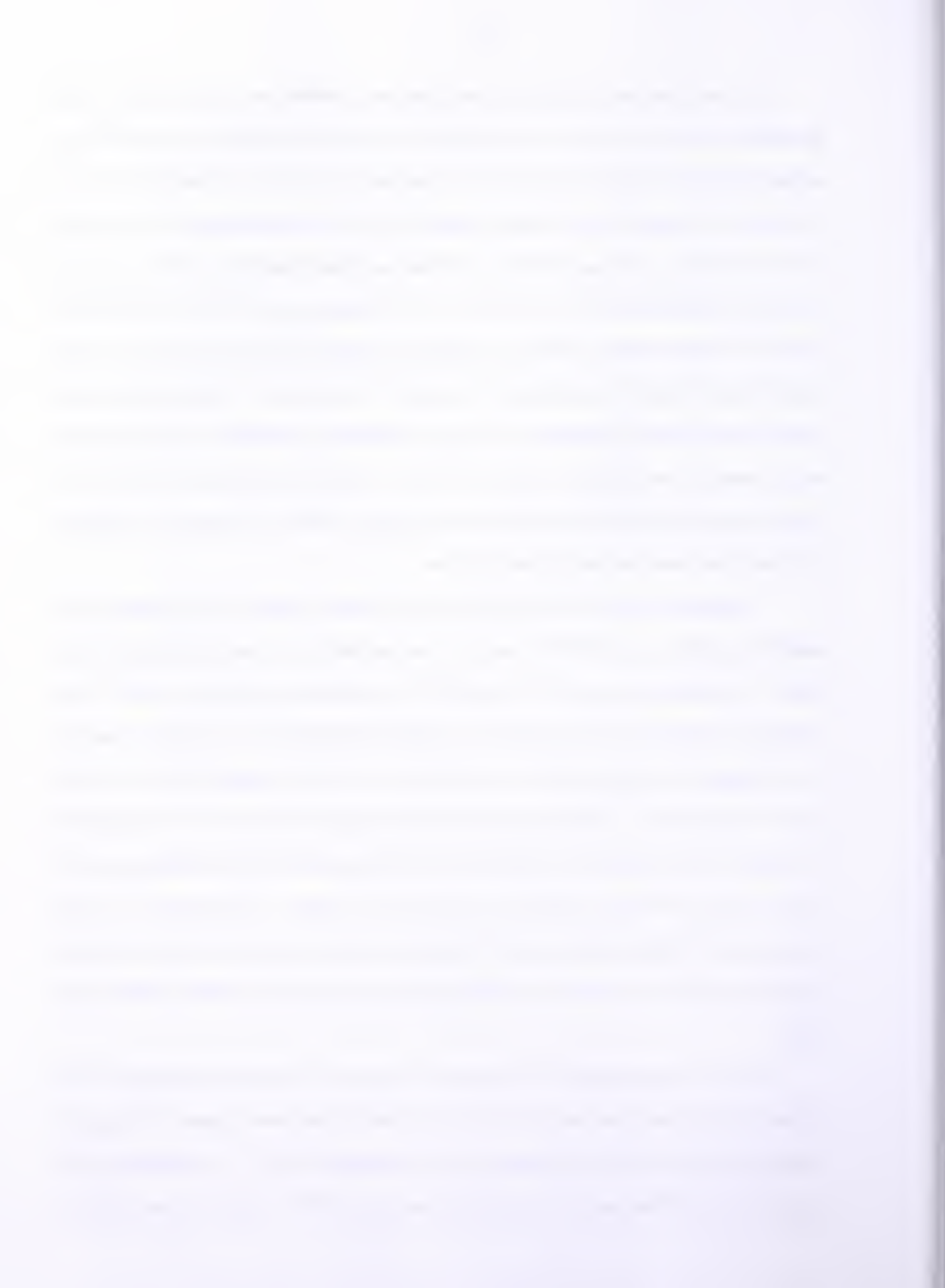




One of the earliest relationships described was between the sense of olfaction and epileptiform attacks. In some of his patients, the eminent neurologist John Hughlings-Jackson observed that some of his patients perceived the presence of a disagreeable smell at the onset of a certain type of seizure. Based on the clinical manifestation of the seizure such as lip smacking, tongue protrusion, sniffing and cheek pouching, Jackson attempted to localize the epileptic foci on the brain. He concluded that based on the physical behavior (or what he called “reflexes”) of the patient during these seizures the most likely brain centers of “epileptic discharge” were those of smell and taste. These reflexes were previously attributed to irritation of the temporo-sphenoidal convolution or the subiculum cornu Ammonis in monkeys (Jackson, 1881). Today these seizures are known as complex partial seizures, (ie: temporal lobe epilepsy), which are believed to originate from the hippocampal-temporal lobe structures.

At around the same time the prominent Baltimore surgeon John Mackenzie was undertaking studies on the effect of smell and the precipitation of coryza and epileptiform attacks. Mackenzie describes the case of a woman whose seizures attacks were sometimes precipitated by the smell of roses (MacKenzie, 1886). As a means of treating her, he exposed the young woman to an artificial rose which precipitated in her a coryza attack without seizures. After several minutes the surgeon explained to her the nature of his therapy. A few days later, realizing that her disease was mostly psychosomatic, the patient came to his office completely cured of her ailments. MacKenzie also gives fascinating historical anecdotes on the influence of odors, especially those from flowers, and their potency in causing severe epileptiform attacks as well as death (MacKenzie, 1886).

Other clinico-pathological disorders involving the olfactory system were well studied by several scientists during the second half of the nineteenth century. Anosmia as a result of a severe blow to the head was a well recognized entity. The mechanisms of injury were well illustrated by Ogle (1870) and Legg (1873). Ogle also described the



relationship between anosmia and the sporadic development of aphasia in several patients. It is interesting to point out that Ogle alludes to a case in which a twelve year old afro-American boy began to lose his skin pigmentation. At the same time, the patient's sense of smell became seriously impaired. He also observed that within mammalian species there was a correlation between darker pigmentation of olfactory membranes and increased acuteness to smell. This led Ogle to conclude that the amount of pigmentation on the olfactory membrane plays an important role in the keenness of scent (Ogle, 1870). Other pathological entities producing anosmia were atrophy, olfactory neuritis, excessive stimulation, inflammation of olfactory nerve, tumors, and diseases of the olfactory centers (Althaus, 1881b). The treatment of choice for anosmia at that time was strychnia applied topically to the mucous membranes, and for hyperosmia a subcutaneous injection of morphine "to narcotize the olfactory nerve" (Althaus, 1881b).

No other system was more closely linked to olfaction than the reproductive system. In an attempt to illustrate the close relationship between the nose and the genitals, MacKenzie outlines several physiological observations (MacKenzie, 1884). First, there was a strong temporal correlation between the engorgement of nasal cavernous tissue and the menstrual cycle in females. Second, in some women who lacked or missed a menstrual cycle this was replaced with nose bleeds. Furthermore, these nose bleeds were also quite frequent in boys reaching puberty. Third, "there is an occasional dependence of genito-urinary irritation upon affections of the nasal passages. Retarded sexual development, too, may possibly depend upon the coexistence of nasal diseases." Fourth there was a close association between nasal congestion or sneezing during periods of sexual arousal (MacKenzie, 1884). Fits of sneezing were also linked to a post-circumcision syndrome (Gray, 1875) and preceding a spontaneous abortion (Mayer, 1875). MacKenzie's physiological associations between the nose and the reproductive system was shared by many scientists. As a consequence, the treatment of



the so called “nasal dysmenorrhea” consisted of cauterization or application of cocaine to distinct areas of the nose identified as the “genital spots” (Ries, 1903).

In addition to physiological associations, Freud paid particular attention to the development of perversions and repression based on the sense of olfaction (Freud, 1985). In his letters to Wilhelm Fliess, Freud explains that:

“...perversions lead to zoophilia and have an animal character. They are explained not by the functioning of erogenous zones that later have been abandoned, but by the effect of erogenous sensations that later lose their force. In this connection one recalls that the principal sense in animals (for sexuality also) is that of smell, which has been reduced in human beings. As long as smell is dominant, urine, feces, and the whole body have a sexually exciting effect. The heightened sense of smell in hysteria presumably is connected with this.”

Freud attempted to illustrate the developmental mechanisms of repression in a similar fashion to the loss of smell during evolution. As human beings began to walk the nose no longer was capable of recognizing interesting sensations found on the earth. The loss of these sensations resulted in the loss of their memory. As a consequence of the loss of their memory a feeling of disgust developed “and in the same manner as we turn away our sense organ (the head and nose) in disgust, the preconscious and the sense of consciousness turn away from the memory. This is repression.” (Freud, 1985).

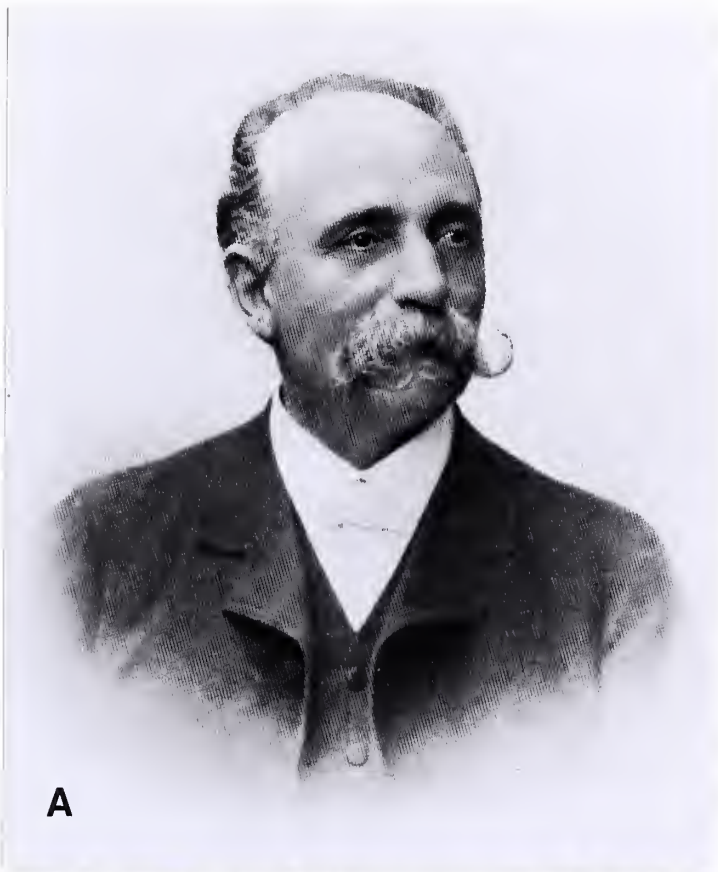
In addition to the clinical manifestations involving the olfactory system, the structural organization and anatomy of this system aided scientists in understanding the organization and function of the nervous system. In 1843 Camillo Golgi (Figure1A) was born in the northern Italian town of Corteno. Excelling at a young age, Golgi decided to follow in his fathers footsteps and enrolled at the Medical School of Pavia University at



Figure 1. (A) Camillo Golgi (1843-1926) (Adapted from Pilleri, 1984). (B) Santiago Ramon Y Cajal (1852-1934) at the age of forty shortly after becoming Professor of Histology in Madrid (Adapted from Ramon Y Cajal, 1937).









the age of sixteen. At the age of twenty one he was appointed professor of pathology and became convinced that clinical problems could be approached by a rigorous understanding of anatomy. In 1872 searching for better pay, he moved to Abbiategrasso which proved to be a bitter disappointment due in part to the poor and dilapidated conditions of the facility. In the kitchen cum laboratory of his institute, under candlelights and very limited resources, Golgi developed a revolutionary technique, “reazione nera” (black reaction), based on a silver nitrate solution. With this new technique Golgi was able to visualize a neuron with its axonal and dendritic extensions. As the neuroscience community has come to know, this reaction had an extraordinary impact in studying the cellular organization of the nervous system. In 1875 Golgi published his classic work on the olfactory bulb (Figure 2) which served as a foundation for his theory on the nerve impulses (Golgi, 1875). Based on the diffuse and complex ramification pattern of neurons, Golgi concluded that all the elements of the central nervous system were intimately linked to form a network. This observation was subsequently designated as “rete nervosa diffusa”. The premise of this theory was that the mode of signal transduction was accomplished by an overall transmission and not by individual units. According to Golgi, a single neuron would only transmit impulses through the axons; the dendrites were not involved in signal transmission. Dendrites performed only a trophic function (Pilleri, 1984, Blanes, 1890). This view was contradicted exhaustively by another famous histologist, Santiago Ramon Y Cajal (Figure 1 B).

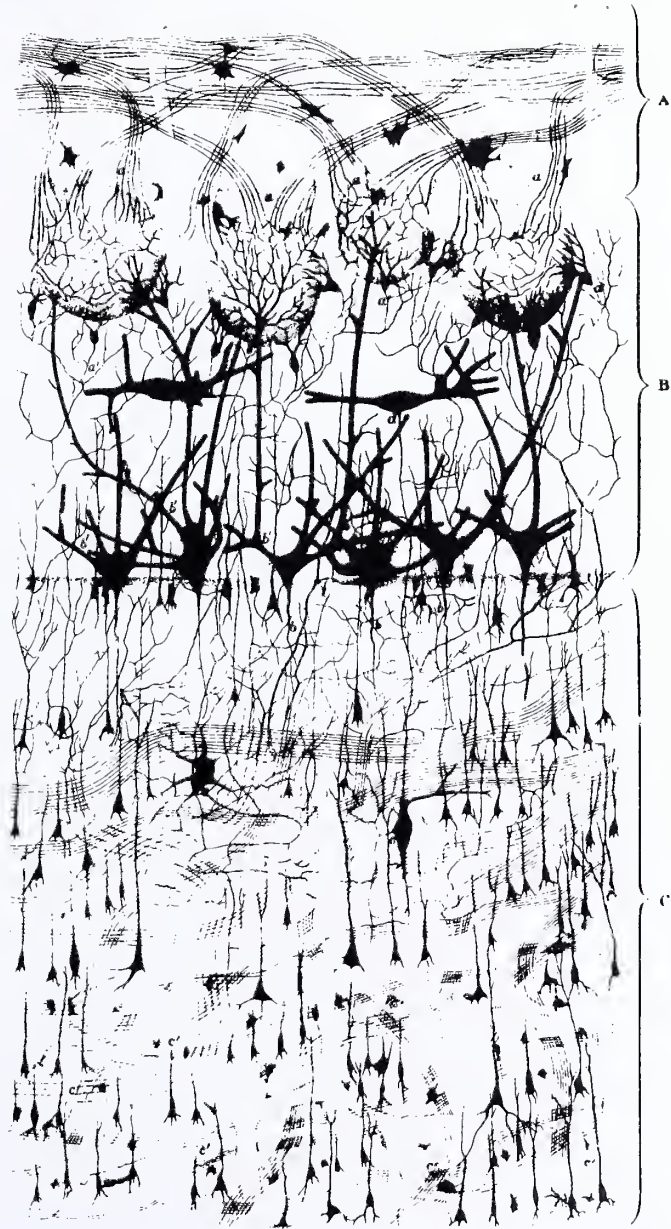
Contrary to Golgi, Cajal’s youth was rebellious and filled with varied ailments including tuberculosis. Cajal’s strength lay in his artistic ability. After several attempts in a variety of careers, his father convinced young Cajal to become a medical illustrator. His interest in histology arose abruptly when one of his teachers showed him the mesenteric blood circulation of the frog. After this experience he realized that a veil has been removed from his face. He bought a small microscope, a Ranvier microtome and





Figure 2. Olfactory bulb laminar structure using the Golgi method. A. Superficial layer of olfactory bulb where olfactory nerve axons originating from the olfactory epithelium terminate. aaa. Olfactory nerve axons penetrating and entering olfactory glomeruli. B. Middle layer where the olfactory glomerulus and the large olfactory neurons (mitral cells) are located. bbb and a'a'a' represent the axons of the mitral and periglomerular cells respectively. b'b'b' are the protoplasmic (dendritic) processes of mitral cells extending tangentially into the glomerulus where they branch extensively. ddd are tufted cells interspersed within the middle layer. C. Internal layer (granule cell layer) where the olfactory tract originates. Within this layer numerous cell bodies of granule cells are found and between these, the mitral and tufted cell axons escape to form the lateral olfactory tract. (Adapted from Golgi, 1875).









began applying the Golgi method with certain practical modifications of his own to study the central nervous system. His initial obstacle, gaining respect from the European scientific community, was overcome when Albert Von Koelliker, the patriarch of German histology, noticed his beautiful preparations of the cerebellum, spinal cord and retina. From this point on Cajal was immediately recognized as a rising star (Pilleri, 1984, Penfield, 1926).

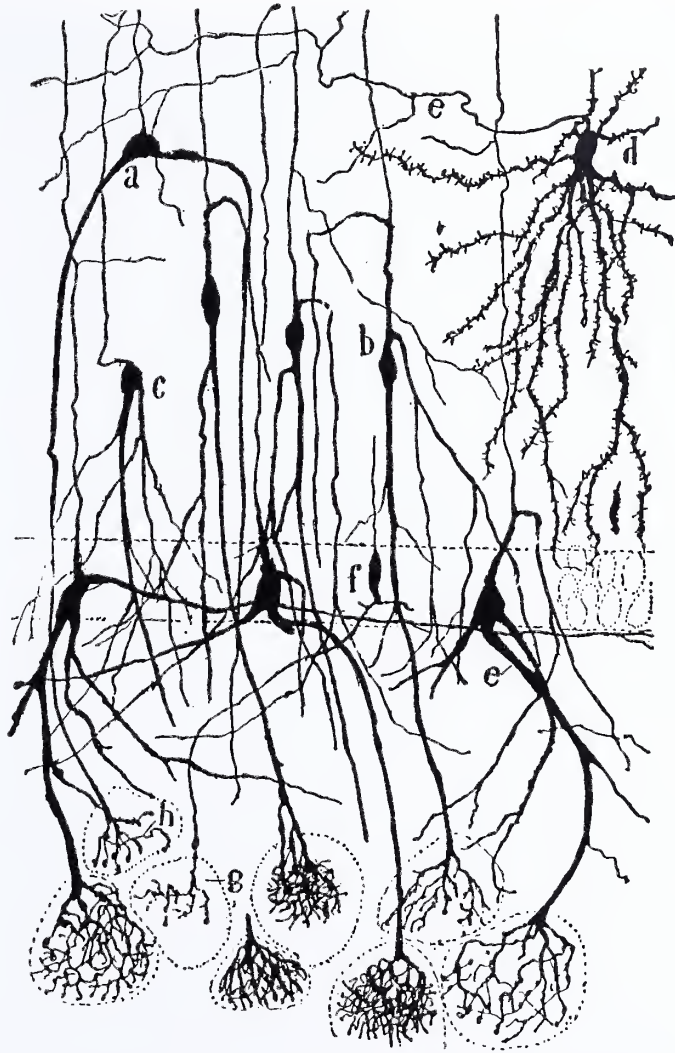
Based on the structural organization and morphology of cells of the retina and olfactory bulb (Figure 3) , Cajal hypothesized that the nerve impulses pass from the dendrites to cell body and from cell body to axon (Addison, 1930). This new concept was coined the dynamic polarity of the neuron. As expected there was an enormous controversy between Cajal's (dynamism) and Golgi's (reticularism) schools of thought . Due in part to its well defined laminar organization, the olfactory bulb became the most appropriate model to support Cajal's theory of dynamic polarity (Blanes, 1890).

In an analogous fashion, the focus of this thesis utilizes the olfactory system as a model to explore the mechanisms that govern the organization and specificity of synaptic connectivity of local circuits in the central nervous system (CNS). The olfactory system serves as an excellent model for this purpose due in part to its easy accessibility and well defined laminar organization. But importantly, it also contains the fundamental components broadly representative of the CNS that make our results applicable throughout the CNS. Finally understanding principles of synaptic specificity and organization may shed some light on the possible role of the olfactory bulb in several clinical diseases (*vide infra*).



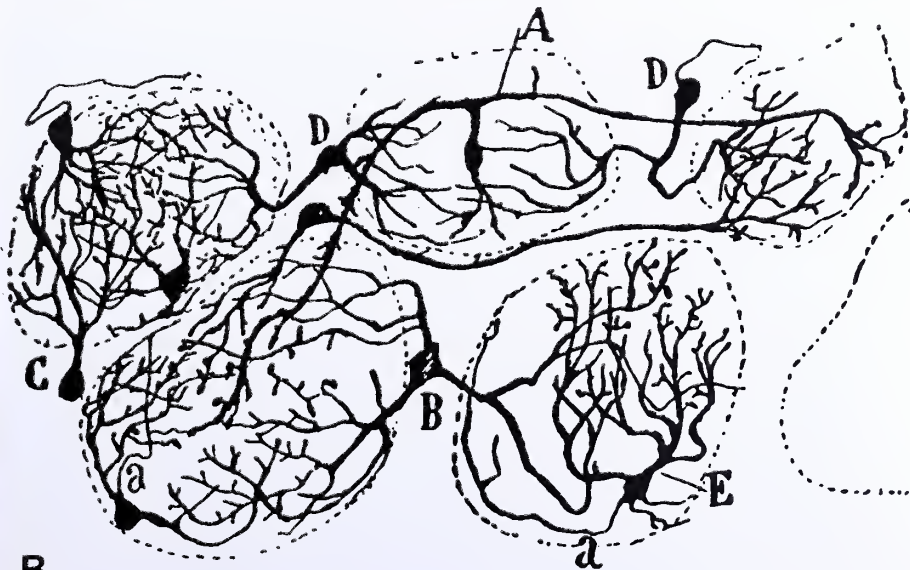


Figure 3. (A) Cajal's illustration of the olfactory bulb using a modified Golgi technique. a,b displaced mitral cells; c, granule cell; d, short axon cells; e, mitral cells; f, small mitral cell; g,h, olfactory bulb glomerulus showing mitral cells dendritic arborization. (B) Peripheral cell (periglomerular) of olfactory bulb glomeruli. A,B,D, biglomerular cells whose dendritic processes bifurcate and arborize onto two glomeruli; C, uniglomerular cells which contains a dendritic arbor restricted to one glomerulus. a, axonal extension from periglomerular cell.



A

FIG. 417. — Quelques cellules du bulbe olfactif du chat. Méthode de Golgi.  
D'après Blanes Viale.



B

FIG. 414. — Grains périphériques des glomérules olfactifs.  
Méthode de Golgi. D'après Blanes Viale.



## B. ANATOMY OF THE OLFACTORY BULB

### 1. Gross Anatomy

The olfactory system in most vertebrates can be divided into three anatomical compartments; 1) the olfactory epithelium, 2) olfactory bulb, 3) piriform cortex (Greer, 1991). The relative size of the olfactory structures correlates closely with the dependency on such a system. For example, the macrosmatic mammals such as rodents and dogs depend on the olfactory system for behaviors that are critical for the survival of the species including alimentation and mating. For this reason, the olfactory bulbs of these animals compromise a large proportion of their brain. On the other hand, the olfactory bulbs of microsmatic mammals, who depend heavily on sight and hearing, occupy a smaller proportion of their brain (McLean and Shipley, 1992; Gilman and Newman, 1987).

In order to gain an overall understanding of odor processing, the pathway followed by odor signals may be briefly summarized as follows (Figure 4 ). Odors traveling within the nose interact with the olfactory receptor cells located in the olfactory epithelium. The axons of the receptor cells pierce the cribriform plate and establish synaptic contact with projection and intrinsic neuron processes located within the glomerular layer of the olfactory bulb. The axons of these projection neurons exit via the lateral olfactory tract and terminate in higher cortical regions such as the piriform cortex, olfactory tubercle, and the amygdaloid complex (Greer, 1991). From these regions further tertiary connections are made that integrate and modify the response of the animal to olfactory information (McLean and Shipley, 1992).

The advantage of studying the olfactory bulbs of rodents are twofold. First, the bulbs are easily accessible as large protrusions arising from the frontal lobes. Second , the olfactory bulbs are organized into distinct laminae with sharply differentiated cell types. This provides a groundwork for understanding the fundamental principles





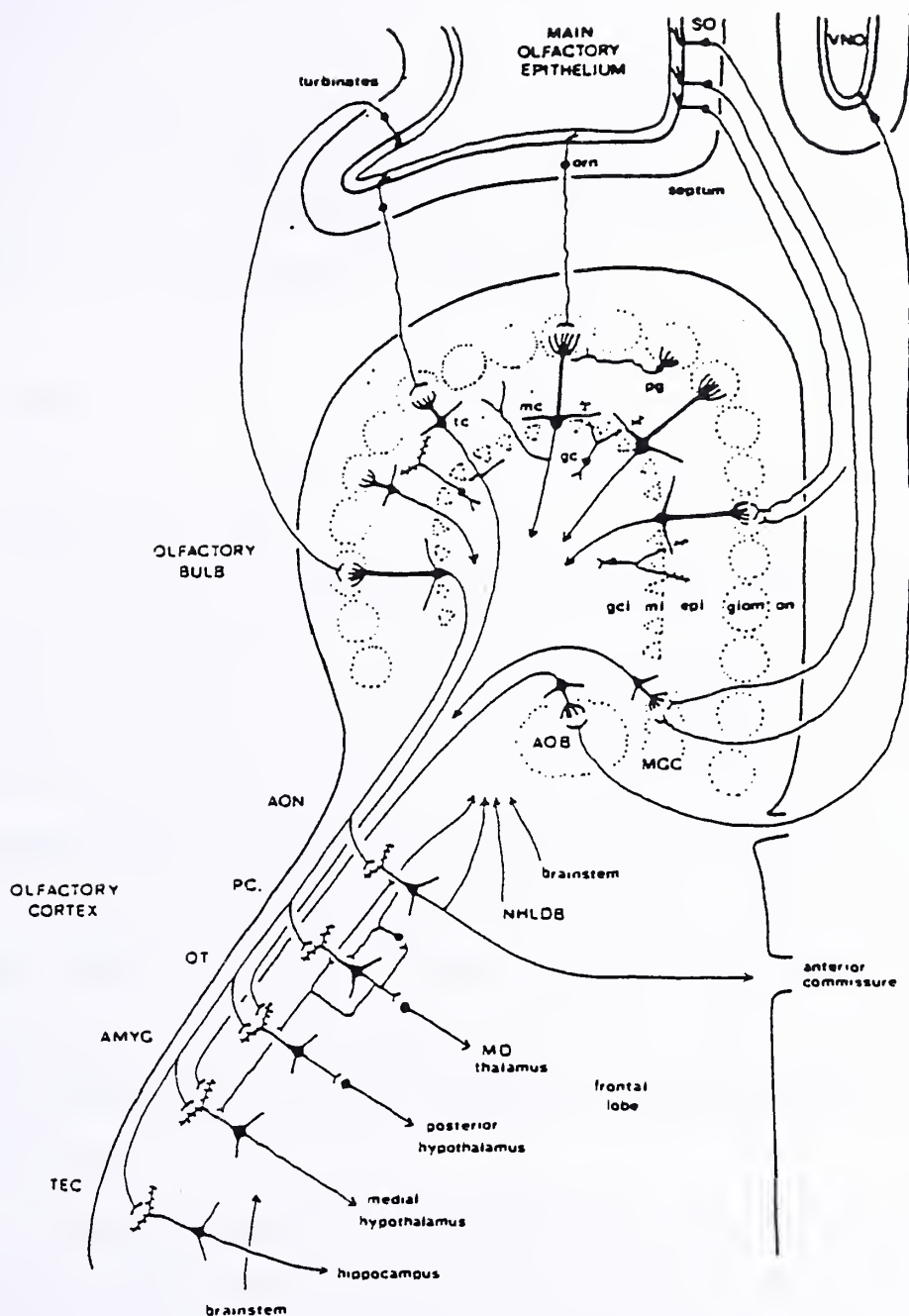


Figure 4. An overview of the olfactory pathway. The olfactory bulb receives input from the receptor neurons in the olfactory epithelium and projects to the olfactory cortex. The diagram indicates some essential aspects of the projection patterns between the regions as well as the main neural elements within the olfactory bulb. Note that the olfactory epithelium is arranged in overlapping populations of receptor neurons which project to individual glomeruli. Some of the central connections to limbic brain structures are also indicated. AMYG, amygdala; AOB, accessory olfactory bulb; AON, anterior olfactory nucleus; MD, mediodorsal; MGC, modified glomerular complex; NHLDB, nucleus of the horizontal limb of the diagonal band; OT, olfactory tubercle; PC, piriform cortex; TEC, temporal entorhinal cortex; VNO, vomeronasal organ; epl, external plexiform layer; gc, granule cell; gcl, granule cell layer; mc, mitral cell; ml, mitral cell layer; on, olfactory nerve layer; orn, olfactory receptor neuron; pg, periglomerular cell; so, septal organ; tc, tufted cell. (Adapted from Shepherd and Greer, 1990.)



governing mechanisms of organization within the CNS. In order to appreciate the convenience of the olfactory system we will begin by discussing the six principal layers (Figure 5 and 6) that compromise the olfactory bulb.

## 2. Layers

### a) Olfactory Nerve Layer

In mammals the outermost lamina of the olfactory bulb is the olfactory nerve layer which contains olfactory receptor cell axons, 0.2 $\mu$ m thick, and glial cells (Shepherd and Greer, 1990; McLean and Shipley, 1992). After piercing through the cribiform plate these axons are partially wrapped by specialized astrocytes called “ensheathing cells” (Doucette, 1988). The olfactory receptor cell axons bundle into discrete fascicles forming a superficial stratified layer of varying thickness along the main olfactory bulb (Halász, 1990). Millions of these axons terminate in specialized cylindrically shaped structures called glomeruli (Blanes, 1890; Allison and Warwick, 1949). Within the glomeruli the axons establish synaptic contacts with intrinsic and projection neurons (Shepherd and Greer, 1990).

Numerous studies have shown a certain degree of topological organization of the projections from the olfactory epithelium onto the bulb (Costanzo and O’Connell, 1980; Jastreboff et al., 1984; Saucier and Astic, 1986; Duncan et al., 1990; Greer et. al., 1991).

### b) Glomerular Layer

Immediately deep to the olfactory nerve layer lies the glomerular layer which is the area of interest in this study. Here, the olfactory receptor axons establish axodendritic synapses onto processes arising from intrinsic neurons (periglomerular cells) and projection neurons (mitral and tufted cells) (Shepherd and Greer, 1990). The glomerular layer is the first tier in olfactory coding processing and it is considered to be the most



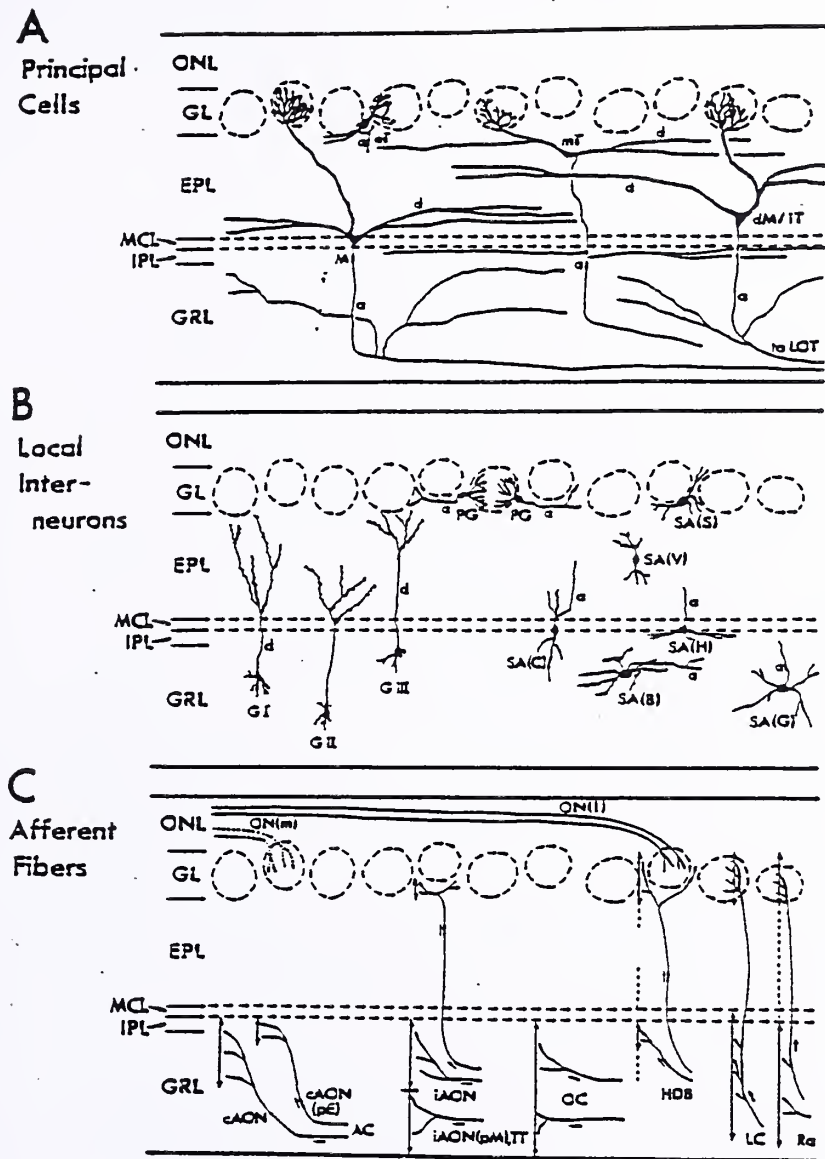


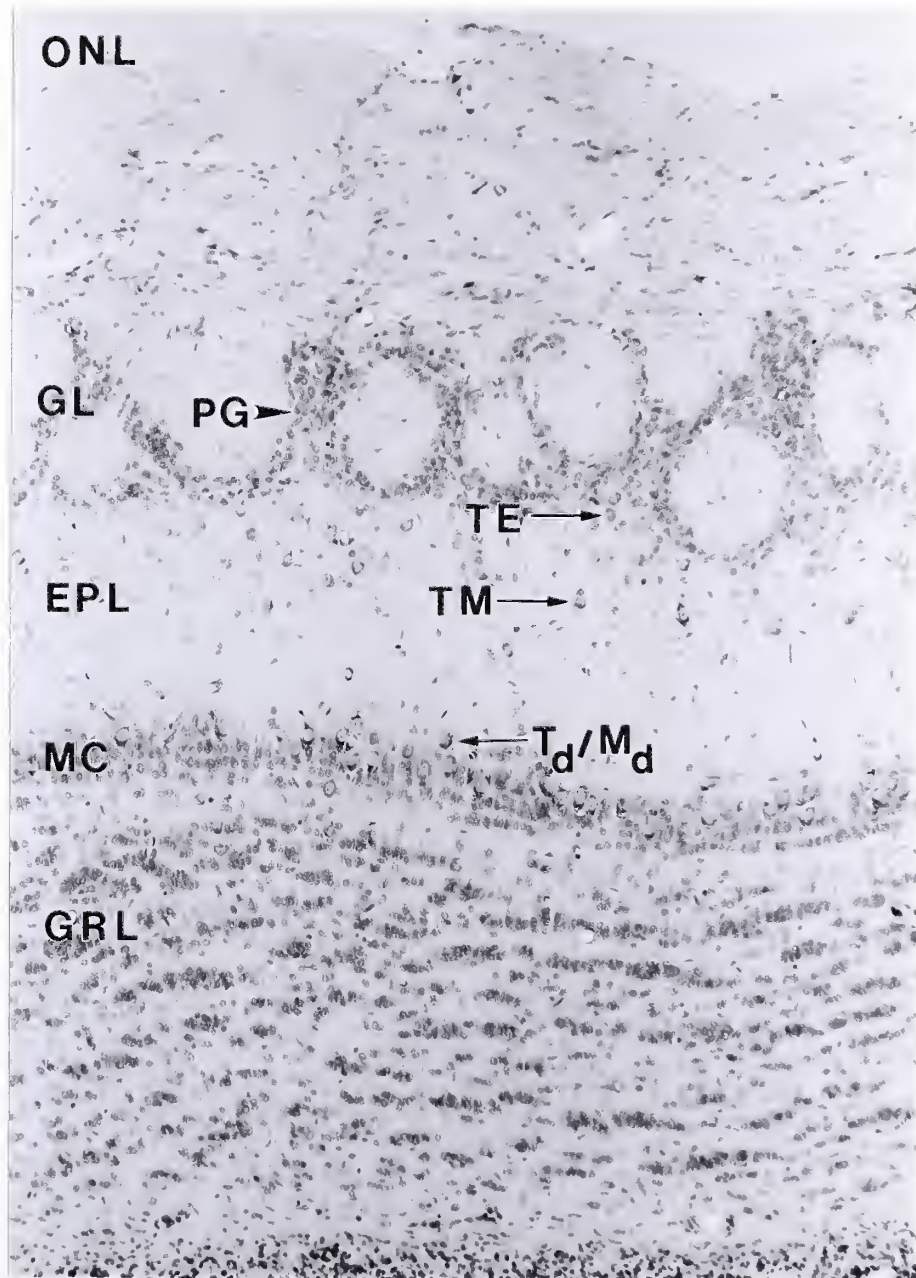
Figure 5. The neural elements of the mammalian olfactory bulb grouped according to the subdivisions into afferent fibers, principal neurons, and intrinsic neurons (local interneurons). Diagram based using the Golgi method. Abbreviations for layers: ONL, olfactory nerve layer; GL, glomerular layer; EPL, external plexiform layer; MCL, mitral cell layer; IPL, internal plexiform layer; GRL, granule cell layer. A. ON(m) and ON(l) indicate medial (m) and lateral (l) subtypes of olfactory nerve fibers. Centrifugal afferents are from the contralateral anterior olfactory nucleus (cAON), ipsilateral anterior olfactory nucleus (iAON), tenia tecta (TT), olfactory cortex, horizontal limb of the diagonal band (HDB), locus coeruleus (LC), and raphe nucleus (Ra); (pE, pars externa of the AON; pM, pars medialis of the AON). B. Dendrites and axon collaterals of a mitral cell (M), and internal tufted cell (T<sub>i</sub> or a displaced mitral cell, M<sub>d</sub>), a middle tufted cell (T<sub>m</sub>), and an external tufted cell (T<sub>e</sub>); a, axon; d, dendrite; LOT, lateral olfactory tract. C. Three types of granule cells (GI, GII, GIII); PG, periglomerular cell; SA, short axon; SA(B), Blanes' cell; SA(C), Clandins' cell; SA(G), Golgi's cell; SA(H), Hensen's cells; SA(S), Schwann cell; SA(V), van Gehuchten cell (Adapted from Shepherd and Greer, 1990).





Figure 6. Photograph of a 10 $\mu$ m thick coronally sliced olfactory bulb stained with cresyl violet showing the laminar organization of the bulb. The five major layers are the: ONL, olfactory nerve layer; GL, glomerular layer; EPL, external plexiform layer; MCL, mitral cell layer; GRL, granule cell layer. Two populations of intrinsic neurons are shown: PG, periglomerular cell; G, granule cell. The primary neurons are the mitral cells (M) and several subpopulation of tufted cell; T<sub>e</sub>, external tufted cell; T<sub>m</sub>, middle tufted cell; M<sub>d</sub> or T<sub>d</sub>, displaced mitral or deep tufted cell. Magnification= 306.25X







distinctive anatomical and physiological unit of the olfactory bulb (Andres, 1965; Shepherd, 1972b; Halász, 1990; McLean and Shipley, 1992). Individual glomeruli are composed of cell free cylindrical structures that are sharply delineated by a surrounding shell composed of periglomerular, short axon, and superficial tufted cell bodies. Depending on the species, these structures range from 30 to 200 $\mu$ m in diameter (Allison, 1953). It is estimated that the number of glomeruli is approximately 2000 in rabbits (Allison and Warwick, 1949) and mice (Allison, 1953; White, 1972; Brunjes, 1983) and in rats it has been estimated at 3000 (Meisami and Safari, 1981). Consequently there is a high convergence of the millions of olfactory axons into a limited set of glomeruli. This raises many potential questions on the mechanisms underlying target specificity which will be discussed in the closing arguments of the introduction.

The principal cells found in the glomerular layer are the periglomerular and short axon cells. The periglomerular cells are a population of intrinsic neurons whose dendritic processes innervate the glomerulus establishing local synaptic circuits with the olfactory receptor axons and mitral/tufted cell dendritic arborization (Pinching and Powell, 1971a,b; White, 1972). In addition to these synaptic components the glomeruli receive limited centrifugal input from several cortical regions such as locus coeruleus, raphe nuclei and the diagonal band of Broca (Mori, 1987; Shepherd and Greer, 1990).

### c) External Plexiform Layer

Immediately adjacent to the glomerular layer is the external plexiform layer. This layer consists mostly of basal dendrites from projection neurons (mitral/tufted cells) and granule cell dendrites forming an intricate network of local circuits. In addition, several axonal projections from cortical centers terminate within the external plexiform layer (Halász, 1990; Reese and Shepherd, 1972). The external plexiform layer also includes the cell bodies of tufted cells which can be divided into superficial, middle and deep according to their relative depth in the external plexiform layer (Orona et. al., 1984).



#### d) Mitral Cell Layer

Deep to the external plexiform layer lies the mitral cell layer. It is only one to two cells thick and is often considered a monolayer. The mitral cell layer is composed mainly of mitral cell bodies with only occasional granule cell bodies (Shepherd and Greer, 1990).

#### e) Internal Plexiform Layer

Lying between the mitral and granule cell layer is the internal plexiform layer. This layer consist mostly of axons from cortical central regions and mitral/tufted cell as well as dendrites of granule cell (Halász, 1990; McLean and Shipley, 1992). Recent immunocytochemical/tracing studies seem to suggest that some of the cortical axons originate from raphe nuclei (McLean and Shipley, 1987) and locus coeruleus (McLean et. al., 1989).

#### f) Granule Cell Layer

The granule cell layer is the deepest neuronal layer of the olfactory bulb and is composed largely of granule cell bodies. These intrinsic neurons are arranged in tightly packed row-like clusters. Interspersed between the cell bodies, the myelinated axons from the tufted/mitral cells gather as they form the lateral olfactory tract. Also included within the granule cell layer are unmyelinated axons arising from cortical centers en route to more superficial layers of the olfactory bulb (Halász, 1990; Shepherd and Greer, 1990; McLean and Shipley, 1992).

### 3. Cell Types

Within the principal layers of the olfactory bulb there are five distinct cell types representative of other areas of the brain . Three are considered to be interneurons responsible for local synaptic circuits. Local synaptic circuits are defined as those whose





connections are confined to a region of the brain and are primarily responsible for information processing. This is in contrast to long distance or projection circuits which transfer information between regions of the brain (Rakic, 1976). The three intrinsic neurons are short axon cells, periglomerular cell and granule cells. The two projection neurons, mitral and tufted cells, send their axons via the lateral olfactory tract to higher olfactory centers (Figure 4 and 5). The morphological features of these cells were previously described by Golgi (1875), Cajal (1890, 1911) (found in figure 2 and 3 respectively) and Blanes (1890). Subsequent studies have elaborated further many subtypes among these populations of neurons.

In addition to their distinct morphological features, the olfactory bulb neurons can be further classified into subtypes dependent on the neuromodulators and neurotransmitters that they express. The following section will present the morphological features of the intrinsic and projection neurons found in the olfactory bulb.

#### a) Short Axon Cells

Short axon neurons are local interneurons with medium size bodies of approximate 8-12 $\mu$ m in diameter. Golgi studies reveal that the dendritic process of these cells are entirely periglomerular and do not receive contact from the olfactory nerve (Shepherd and Greer, 1990; McLean and Shipley, 1992). Based on their laminar distribution and morphology of their cell bodies these neurons can be subdivided into six subtypes. These cells have not been well characterized and will not be discussed further.

#### b) Periglomerular Cells

Periglomerular cells are small (5-8 $\mu$ m) neurons located at a high density surrounding glomeruli. In very thorough studies Pinching and Powell (1971a, 1971b) determined that the dendrites of these cells penetrated more than one but preferentially one glomerulus usually extending 50-100 $\mu$ m. These dendrites appeared not to fill the





entire glomerulus, but they were organized in a highly well defined structural fashion. At the electron microscopic level these intrinsic neurons were seen establishing synaptic contact with presumed mitral/tufted cells and receiving synaptic input from the olfactory nerve and mitral/tufted cell processes . The axons of these neurons are restricted to intraglomerular regions extending laterally to about 800 $\mu$ m long (Pinching and Powell, 1971a, 1971b; White, 1972). The periglomerular cells are not differentiated into subtypes based on their morphology. However, there are distinct subpopulations based on the neurotransmitters they utilize (see below).

### c) Granule Cells

As mentioned previously, granule cell are found in the deepest layer of the olfactory bulb, the granule cell layer. Granule cells are often compared to the amacrine cell of the retina based on their morphology and presumed physiologic role in local circuits. Granule cells are slightly larger than periglomerular cells (6-8 $\mu$ m) and are axonless neurons (Cajal, 1890, 1911; Price and Powell, 1970a,b; Schneider and Macrides, 1978). Another interesting characteristic of these cells is the high density of spines along their dendritic arborization (Greer, 1987, Woolf et. al, 1991a,b). The dendritic processes of granule cells are divided into apical segments, which extends radially from the somata into the external plexiform layer, and a basal component that remains confined to deeper parts of the granule cell layer. The apical dendrites are believed to establish synaptic contact with mitral and tufted cells thus, processing signal output (Shepherd and Greer, 1990; Woolf et. al. 1991a,b). Based on the differential distribution of their apical dendrites and location of their somata within the granule cell layer, the granule cells have been divided into three subtypes (Orona et. al., 1983; Mori et. al, 1983; Greer, 1987).

Superficial granule cell bodies are closer to the mitral cell layer and extend their apical dendrites eventually branching at the most superficial portions of the external plexiform layer. Deeper granule cells, on the other hand, are found in the deeper aspects



of the granule cell layer and extend their dendrites through this layer ultimately branching in deepest portions of the external plexiform layer. As expected, the third type, the intermediate granule cells, have their dendritic process contained in between the aforementioned cell types. These specific patterns of selective dendritic arborization raises the notion of potential parallel circuits for odor processing information (Greer, 1991). In addition to these morphological differences, Struble and Walters (1982) identified two subpopulations of granule cells based on their staining pattern using toluidene blue.

#### d) Mitral Cells

Named after their resemblance to a bishop's mitre, the mitral cells are the principal neurons of the olfactory bulb. Located in the mitral cell layer, their cell bodies range from 15 to 35 $\mu$ m in diameter. An apical dendrite arises from the somata and reaches a single glomerulus without emitting side branches. Within the glomerulus the dendritic processes arborize extensively and establish synaptic contact with the olfactory nerve and intrinsic neurons. The dendrites of approximately 25 mitral cells enter each glomerulus (Cajal, 1911; Allison and Warwick, 1949). A single mitral cell tends to emit 2 to 9 secondary dendrites which branch several times within the external plexiform layer. These secondary dendrites tend to run parallel to the surface of the bulb, extending up to 2mm in the external plexiform layer, and establish synaptic contacts with dendrites of granule cells (Rall et. al., 1966; Macrides and Schneider, 1982; Orona et. al., 1983; Nickell and Shipley, 1992).

At least two subpopulations of mitral cells have been identified based on their dendritic arborization. The M<sub>1</sub> mitral cells tend to have their secondary dendritic processes in the deeper portions of the external plexiform layer, while the M<sub>2</sub> subtype arborizes superficially in the external plexiform layer adjacent to the glomerular layer (Macrides and Schneider, 1982; Mori et. al. 1983; Orona et al., 1984). The axons of



mitral cells arise from the deep side of the somata and run into the granule cell layer where they branch into collaterals. Contrary to Cajal's (1911) and Allison's (1953) observation, these axonal collaterals do not escape the granule cell layer into the external plexiform layer. The main axons of the mitral cells terminate in all the target areas of olfactory cortex (Kishi et al., 1984; Orona et al., 1984; Haberly and Price, 1977).

#### e) Tufted Cells

Tufted cells are one of the primary projection neuron of the olfactory bulb with somata ranging in size between 15 to 20 $\mu$ m. Initially considered by Cajal (1890) as dislocated mitral cells, it is well known today that tufted cells have a distinct morphological pattern (Cajal, 1911, 1955; Macrides and Schneider, 1982; Mori et. al. 1983; Kishi, 1987; Orona, 1984) and genetic origin (Greer and Shepherd, 1982; Mullen et. al, 1976). Their cell body size differs depending on their location within the external plexiform layer. The deeper their cell bodies are within the external plexiform layer the larger their cell body. Based on the size and differential dendritic arborization tufted cells are divided into deep, middle and superficial (Cajal, 1911, 1955; Macrides and Schneider, 1982; Mori et. al. 1983; Kishi, 1983; Orona, 1984).

Deep tufted cells are scattered sparsely in the deeper one third of the external plexiform layer. The dendritic distribution of these cell is similar to that of mitral cells ( $M_2$ ) type, but differ slightly in the pattern of dendritic distribution and extent (Macrides and Scneider, 1982). Middle tufted cells have a medium size somata in the superficial two thirds of the external plexiform layer. They typically extend a single apical dendrite that arborizes into specific domains in a single glomerulus . The primary dendrites of middle tufted cells tend to project in a tangential manner throughout the external plexiform layer. The secondary dendrites project mostly into the superficial part of the external plexiform layer (Macrides and Scneider, 1982). The axons of these cells often have extensive bifurcation in the internal plexiform layer (Greer, 1991). The axons of





middle and deep tufted cells, that are not part of the intrabulbar associational system, coalesce and form the lateral olfactory tract. The projections from the tufted and mitral cell differ in their axon collateral patterns and projections to higher olfactory centers . The extent of the olfactory cortical areas that tufted cell project to roughly correlates to their laminar position in the external plexiform layer. Thus, deep tufted cells project extensively to virtually all areas of the olfactory cortex. Meanwhile, middle tufted cells project to only the more rostrally located olfactory centers (Haberly and Price, 1977; Skeen and Hall, 1977; Scott, 1981).

The superficial tufted cells are small sized neurons distributed relatively densely in the periglomerular region between the glomerular and external plexiform layer. Many of them have dendritic processes that extend into a single glomerulus, while a few innervate two glomeruli (Pinching and Powell, 1971a; Schneider and Scott 1983). The axons of the superficial tufted cells are mostly restricted to the intra-olfactory bulb associational system (Macrides et. al., 1985).

#### 4. Synaptic Organization

Since the focus of this thesis is on the ultrastructural organization of the glomerular layer, the following is a brief description of the synaptic organization of the olfactory bulb (Figure 7). The basic organization of the olfactory bulb has been used as a model system for understanding intrinsic neuronal elements and their corresponding synaptic organization throughout the central nervous system (Shepherd and Greer, 1990).

The synaptic organization of the glomerular layer can be divided into intraglomerular and interglomerular connections. Prior to entering the glomerular layer the receptor cells axons are organized into very well defined fascicles. Once these axons reach the glomerulus there is a segregation and reorganization of these axons into specific glomeruli and within a glomerulus the axons appear organized into specific subcompartments. The olfactory receptor cell terminals are characteristically electron





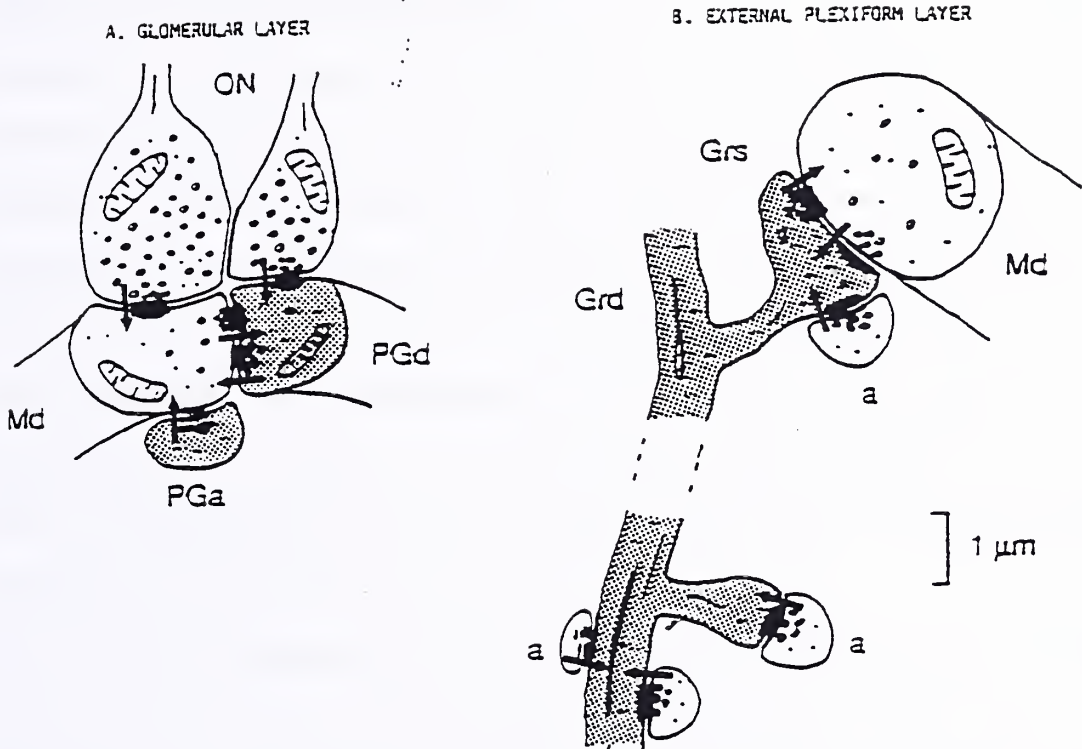


Figure 7. Synaptic circuit organization of olfactory bulb. The presynaptic inhibitory components are shaded; excitatory components are not shaded. A. Synaptic connections in the glomerular layer. Two olfactory receptor cell axons (ON) synapse onto mitral/tufted dendrite (Md) and a periglomerular cell dendrite (PGd). The two dendrites are also in reciprocal synaptic contact. A periglomerular cell axon (PGa) from a distant cell synapses onto the mitral/tufted cell dendrite. B Synaptic connections in the external plexiform layer. A granule cell spine (Grs) is in reciprocal dendrodendritic synaptic contact with a mitral/tufted cell dendrite. The spine is seen emerging from the parent granule cell dendrite (Grd). Centrifugal axons (a) are seen synapsing on the granule cell processes. Arrows indicate direction or polarity of synapse. (Adapted from Shepherd and Greer, 1990).



dense with many small spherical vesicles filling the axoplasm. In the intraglomerular connections the receptor cell axons establish an axodendritic synaptic contact with presumed mitral/tufted and periglomerular cells dendritic processes. These synapses are type-I synapses which are characterized by numerous round vesicles in the axoplasm juxtaposed to a thick asymmetrical postsynaptic membrane specialization on the receiving dendritic process. It is not known if these axons establish a homogenous distribution of synaptic connections, that is, if an individual axon contacts only mitral/tufted or periglomerular cells, or rather distributes uniformly to all processes. A situation of non-uniform distribution has been raised by White (1972) where through serial reconstructions of the olfactory glomerulus he describes a strain of mice (BALB/c) missing the synaptic connection between the receptor cell axons and the periglomerular cells. No evidence has been found suggesting that receptor cell axons receive conventional synaptic contact from any source.

The other component of intraglomerular connections consists of the mitral/tufted cells establishing contacts with periglomerular cell processes. This contact can be categorized as a reciprocal synapse in which (Figure 7 A) the mitral/tufted cell establishes a type I synapse onto periglomerular cell, while the periglomerular cell makes a type II synapse onto the mitral/tufted cell process. Type II synapses are characterized by numerous pleomorphic vesicles in the periglomerular process juxtaposed to a symmetrical postsynaptic specialization (Hirata, 1964; Pinching and Powell, 1972; Getchell and Shepherd, 1975). The reciprocal pair are hard to see in single sections, however, with serial reconstructions it has been shown that approximately 25% of these pairs of synapses are arranged side by side (Shepherd and Greer, 1990). Within the glomerulus limited centrifugal fibers, such as those from the raphe nucleus are also seen to establish synaptic contacts (Pinching and Powell, 1972; Mori, 1987). The complex synaptic arrangement described above may serve as a signal modulator and processor for incoming odor information.



The interglomerular synaptic organization is composed of axons from periglomerular cells traveling to other glomeruli and establishing extra-glomerular type-II synapses onto apical dendrites of mitral/tufted cells and other periglomerular cell bodies (Pinching and Powell, 1971a,b). Also present are type-I synapses between superficial tufted cell axons and tufted cell dendrites as well as presumed centrifugal axons synapsing onto periglomerular cell bodies (Pinching and Powell, 1971a,b, 1972).

The next tier containing local microcircuits is the external plexiform layer (Figure 7 B). In this layer the dendritic processes of granule and mitral/tufted cell establish synaptic contact in a manner similar to that seen in the glomerulus. At the most superficial layer the dendritic spines of superficial granule cells establish contact with the dendritic processes of tufted cells. Deeper, the dendritic spines of deep granule cells makes synaptic contact with the secondary dendrites of mitral cells. At the level of the electron microscope the dendritic processes of mitral and tufted cells appear as large, electron lucent, tangentially oriented through the external plexiform layer with a distribution of microtubules and several organelles. These electron lucent process are seen establishing type I, asymmetrical synapses onto granule cell spines (gemmules) (Rall et al., 1966; Rall and Shepherd, 1968; Jackowski et al., 1978). In turn, the relatively electron dense granule cell spines establish type-II, symmetrical synapses onto the dendritic processess of mitral/tufted cells. The neurotransmitter mediating the granule to mitral/tufted synapses is GABA while conventional data suggest glutamate is utilized by mitral cells (Phillips et al., 1963). The reciprocal synapses in this layer are found to exists in a dynamic equilibrium of 1:1 (Woolf and Greer, 1991a). This is supported by developmental studies where the asymmetrical synapse appeared first followed by the reciprocal symmetrical synapse (Hinds and Hinds, 1976a,b) and in a genetic mutant where denervated granule cell spines formed new reciprocal synapses with tufted cell dendrites (Greer and Halász, 1987). Furthermore, it is interesting to note that the pattern of organization of granule cell spines is nonrandom. Implementing serial reconstructions





Woolf and Greer (1991) were able to demonstrate that the spines organize in a columnar fashion along the mitral/tufted cell dendrites.

In addition to the dendrodendritic connections, within the external plexiform layer there are axodendritic connections from short cell axons and central projections (Mori, 1987). The central projections arise from the horizontal limb of the diagonal band, the nucleus raphe, piriform cortex and anterior olfactory nucleus. The axodendritic connections are believed to be involved in inhibition of mitral/tufted cells through the excitation of granule cells (Shepherd and Greer, 1990).

Lastly, the synaptic organization of the mitral-granule cell layers consists of axon collaterals of mitral/tufted cells terminating on the basal dendrites of granule cells. These synaptic terminations are believed to be excitatory in nature which in turn inhibit the mitral/tufted thus further modulating the odor signal output (Price and Powell, 1970b,c). Within the granule cell layer there are also terminating fibers from central areas such as those arising from the horizontal limb of the diagonal band and anterior olfactory nucleus.

## 5. Neurochemistry

The process of exchanging information between two neurons could be through an electrical or chemical synapse. Characteristics of electrical synapses are their fast speed of action and inability to produce long lasting changes. These electrical synapses are structurally dependent on gap junctions for transmission of ionic current thus mediating electrical conductivity. For this reason electrical synapses could be either unidirectional or bi-directional as well as synchronous. Most of the synapses found in the brain are chemical in nature. Structurally, chemical synapses are composed of a pre- and postsynaptic specializations separated by a synaptic cleft. Although this physical separation attenuates the speed of transmission, these synapses have the capability of signal amplification by releasing thousands of synaptic vesicles. Another advantage of





chemical synapses is the capability of enduring changes in effectiveness. This plasticity is important to memory and other higher cognitive functions. Depending on the receptor, chemical synapses can act as either inhibitory or excitatory (Schwartz, 1991) .

The concept of a chemical transmitter was first revealed by Otto Loewi in the 1930's who demonstrated the release of acetylcholine from vagus terminals in the frog's heart. Since then several modifications of what classifies as a neurotransmitter have been suggested. Incorporating Kruk and Pycock (1991), and Schwartz (1991) a neurotransmitter has to meet the following criteria: 1) It is synthesized by the neuron. 2) It is present in the presynaptic terminal and it is released in amounts sufficient to exert its supposed action on the postsynaptic neuron or effector organ. 3) When applied exogenously (as a drug) in reasonable concentrations, it mimics the action of the endogenously released neurotransmitter (for example, it activates the same ion channel or second messenger pathway in the postsynaptic cell). 4) A specific mechanism exists for removing it from its site of action (the synaptic cleft). A neuromodulator is a substance which act on or within neurons to alter the responsiveness of the neuron to the effect of a neurotransmitter. This capacity could be done by affecting the synthesis or release of the neurotransmitter or by affecting the sensitivity of the postsynaptic neuron either directly or indirectly (second messengers). The following is a brief description of putative neurotransmitters/neuromodulators found in the neuronal elements of the olfactory bulb.

#### a) Periglomerular Cells

Many of the periglomerular cell are dopaminergic (Halász et. al., 1981a; Davis and Macrides, 1983) or contain gamma-amino-butyrate-acid (GABAergic) (Ribak et. al., 1977). In hamster approximately 70% of dopaminergic periglomerular cells colocalize GABA, and 45% of GABAergic cells colocalize dopamine (Kozaka et. al., 1985). In rat there also appears to be evidence of colocalization of both GABA and dopamine. Approximately 69% of periglomerular cells colocalized GABA and dopamine. Twenty



seven percent were reactive to GABA alone, and the majority of dopaminergic periglomerular cells colocalize GABA (Gall et. al, 1987). Substance P has been reported to be also present on GABAergic and dopaminergic periglomerular cells. Thus some periglomerular cells may contain an inhibitory neurotransmitter, GABA, a catecholamine, dopamine, and a potential excitatory neurotransmitter, substance P (Kosaka et. al., 1988). In addition another group has reported the presence of met-enkephalin (Davis et. al, 1982) and vasoactive intestinal peptide (VIP) (Gall et. al., 1986; Sanides-Kohlrausch, and Wahle, 1990) in rat olfactory bulb periglomerular cells. Also reportedly present in the periglomerular cell are NADPH-diaphorase (Scott et. al, 1987), cholecystokinin (Seroogy et. al, 1985), aspartic acid (Halász, 1987), thyrotropin-releasing hormone (Tsuruo et al., 1988), and protein kinase C (Saito et. al, 1988).

As noted there are several subpopulations of periglomerular cells based on their neurotransmitters. However, little is known about their physiological role in information processing. This difficulty is attributed, in part, to their small size and the limited extent of their local dendritic circuitry.

#### b) Granule Cells

Most of the granule cells contain the inhibitory neurotransmitter GABA (Nicoll, 1971; Ribak et. al., 1977; Halász et. al., 1978) which inhibits the mitral and tufted cells (Shepherd, 1972b; Jahr and Nicoll, 1982). Met-enkephalin has also been localized in granule cells, (Bogan et. al., 1982; Davis et. al. 1982) although the physiological importance of this neuromodulator in odor processing is not known.

#### c) Mitral Cells

The most likely neurotransmitter of mitral cells are the excitatory amino acids glutamate and/or aspartate. This is in part supported by an extensive literature (Halász and Shepherd, 1983; Cotman, 1987; Fuller et al., 1988) but some controversy over this



assumption has been raised (Hori et al. , 1982; Krammer et al., 1980). Another proposed neurotransmitter that has raised some controversy is N-acetyl-aspartyl-glutamate (NAG) which has been exclusively found by immunocytochemistry in mitral cells (Blakeley et al., 1987). However there are some doubts regarding its physiological role as a neuromodulator (Whittemore and Koerner, 1989). Utilizing in situ hybridization, another peptide localized in the mitral cells is corticotrophin-releasing factor (CRF) (Imaki et al., 1989). Once again the physiologic role of CRF remains to be discovered.

#### d) Tufted Cells

The neurotransmitters present in tufted cells are believed to be those of mitral cells. However there are studies that reveal the presence of dopamine, especially on superficial tufted cells (Halász et. al., 1983; Gall et. al., 1987). Also, substance P (Burd et. al, 1985; Baker, 1986), VIP (Gall et al. 1986) and CCK (Seroogy, 1985) have been found in subpopulations of tufted cells . The functional significance of these neuromodulators remains to be established.

Figure 8 represent a summary diagram of the cells just described with their putative neurotransmitter/neuromodulators.

### 6. Functional Organization of Odor Processing

Odor signals from the olfactory epithelium are carried through the receptor cell axons to the olfactory bulb glomeruli. Within the glomeruli the receptor axons establish excitatory axodendritic contact with principal (mitral/tufted) cell dendrites and intrinsic (periglomerular) cell dendrites (see above). The topographical distribution between the olfactory epithelium and bulb as well as the synaptic distribution of the receptor cell axons remains to be established. Following the initial interaction between the sensory afferents and the primary/intrinsic neuronal elements, further odor processing takes place within the glomerulus through dendrodendritic microcircuits (refer to Figure 9). The



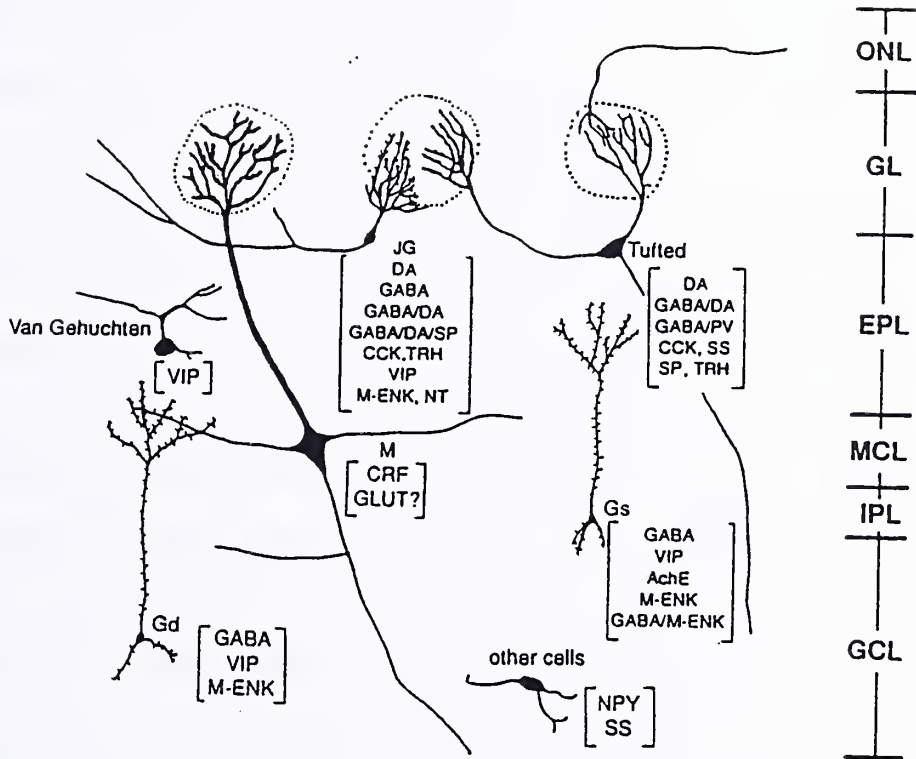


Figure 8. Diagram depicting some of the major neuronal types making up the olfactory bulb with their putative neurotransmitters/neuromodulators. ONL, olfactory nerve layer; GL, glomerular layer; EPL, external plexiform layer; MCL, mitral cell layer; IPL, internal plexiform layer; GCL, granule cell layer; JG, juxtglomerular-periglomerular; M, mitral cell; Gd, deep granule cell; Gs, superficial granule cell; GABA, gamma-amino-butyrate-acid; DA, dopamine; SP, substance P; CCK, cholecystinin; M-ENK, met-enkephalin; VIP, vasoactive intestinal peptide; TRH, thyrotropin-releasing hormone; CRF, corticotrophin-releasing factor; GLUT, glutamate; PV, parvalbumin; SS, somatostatin; AchE, acetylcholine. (Adapted from McLean and Shipley, 1992.)





mitral/tufted cell dendrites establish an excitatory asymmetrical synapses onto periglomerular cell dendrites. In turn the periglomerular cell dendrites control the output signal of the mitral/tufted cells through a reciprocal inhibitory symmetrical synapse (Shepherd and Greer, 1990). Communication between adjacent glomeruli is established through the axons of periglomerular cells making synaptic contact onto mitral/tufted apical dendrites or periglomerular cell bodies (see above).

Several converging lines of evidence suggest that the glomerulus serves as a heterogeneous functional unit for odor processing. Anatomical tract-tracing studies employing horse-radish peroxidase (HRP) into the olfactory epithelium showed variable patterns of staining among and within glomeruli suggesting subglomerular organization for odor processing (Stewart and Pedersen, 1987). This concept was supported in part by physiological studies where glomeruli were shown to have distinct specificities to different odor stimulation (Levetau and MacLeod, 1966). In addition, following odor stimulation metabolic studies using high-resolution 2-deoxyglucose revealed dramatic differences in metabolic activity across the glomerular layer (Greer et al., 1981; Lancet et al., 1981, 1982; Benson et al., 1985). Moreover, studies employing early intermediate genes such as cFOS have shown variable patterns of expression among and within glomeruli after odor stimulation (Guthrie et al., 1993). These observations suggest that glomeruli can be grouped into specific functional odor domains. If a glomerulus has a defined functional specificity, then it is possible that all the mitral/tufted and periglomerular cell dendrites connected to a glomerulus will share the same specificities. This implies that within the olfactory bulb there is a horizontal or columnar functional organization similar to those seen in other areas of the brain such as the cerebellum (Shepherd, 1972a; Shepherd and Greer, 1990).

The next level of odor processing occurs in the external plexiform layer. The main type of microcircuit is the reciprocal dendrodendritic synapse between mitral/tufted cells and granule cells. This was the first dendrodendritic circuit described in the central



nervous system (Rall et al., 1966). In the reciprocal synapses, the mitral-to-granule synapse is excitatory and the granule-to-mitral is inhibitory (Mori and Takagi, 1978; Shepherd and Greer, 1990). As previously noted the secondary dendrites of mitral/tufted cell expand through long distances along the olfactory bulb. Therefore, the overall function of the intricate synaptic connectivity of the external plexiform layer is to provide an inhibitory mechanism for mitral/tufted cells which confines lateralization of odor information thus controlling the output signal (Shepherd and Greer, 1990; Woolf et al., 1991a,b). This lateral inhibition can also serve as a mechanism to enhance differences between active and inactive sites in the bulb (Nickell and Shipley, 1992).

Within the external plexiform layer there is evidence to suggest a sublaminal distribution of secondary dendrites from tufted and mitral cells (refer to Figure 9). Secondary dendrites of middle and superficial tufted cells arborize extensively in the superficial regions of the external plexiform layer. This preferential distribution in the superficial part of the external plexiform layer suggests that these dendrites establish dendrodendritic synaptic contact with superficial granule cells. On the other hand, mitral ( $M_1$  subtype) and deep tufted cells appear to establish dendrodendritic contact with deep granule cells. A third subpopulation of granule cells appears to interact with both mitral and tufted cells. This sublaminal distribution adds further support to the proposal of parallel circuits and columnar arrangement within the bulb for processing odor information (Orona et al., 1984; Greer and Halász, 1987; Mori, 1987; Greer, 1991).

In addition to the intricate local synaptic microcircuitry in the olfactory bulb, odor processing is influenced by higher cortical regions of the brain. The axons of these centrifugal fibers arise from the horizontal limb of the diagonal band, the nucleus raphe, piriform cortex and anterior olfactory nucleus, and terminate mostly in the external plexiform layer and glomerular layer. The axodendritic terminations in the external plexiform layer are excitatory to granule cells, thereby inhibiting through another feedback loop the mitral/tufted cell output. The only source of output for the granule



cells are those in the form of dendrodendritic synapses found on the external plexiform layer (Price and Powell, 1970b, c; Shepherd and Greer, 1990). Centrifugal axons that reach the glomerular layer are mostly restricted to the interglomerular space. Here the centrifugal axons establish excitatory contact with periglomerular cell bodies. Serotonergic fibers from the raphe nucleus enter and arborize within the glomerulus (Pinching and Powell, 1971a,b). The function and synaptic connectivity of centrifugal fibers in the glomerulus to this date is not well understood. The main function of centrifugal fibers into the olfactory bulb is known to serve a significant modulatory influence in odor processing, however, the mechanisms and control of these influences remain to be elucidated. Figure 9 summarizes the basic circuit organization of the olfactory bulb.

### C. IMMUNOCYTOCHEMISTRY

The laminar organization and the heterogeneous population of neurons, (see above) makes the olfactory bulb an optimal model to study issues regarding cellular organization and synaptic connectivity. Given an apparent functional and structural subcompartmentalization of the glomerulus, this study will employ immunocytochemical and anatomical techniques in an attempt to outline mechanisms of synaptic organization in the olfactory bulb glomerulus (see below under Current Study). In particular our attention will focus on periglomerular cell processes immunoreactive for antibodies to tyrosine hydroxylase and GABA. To have a better appreciation for this study, the process of immunocytochemistry will be briefly described followed by a review of dopamine and GABA as neurotransmitters in the central nervous system.

Immunocytochemical labeling uses the antigen-antibody interaction in an attempt to demonstrate the cellular organization of tissues. There are three immunocytochemical methods employed today, the direct method, two step method, and the three step method (Figure 10). Coons et al. (1941, 1942) were the first investigators to introduce the direct





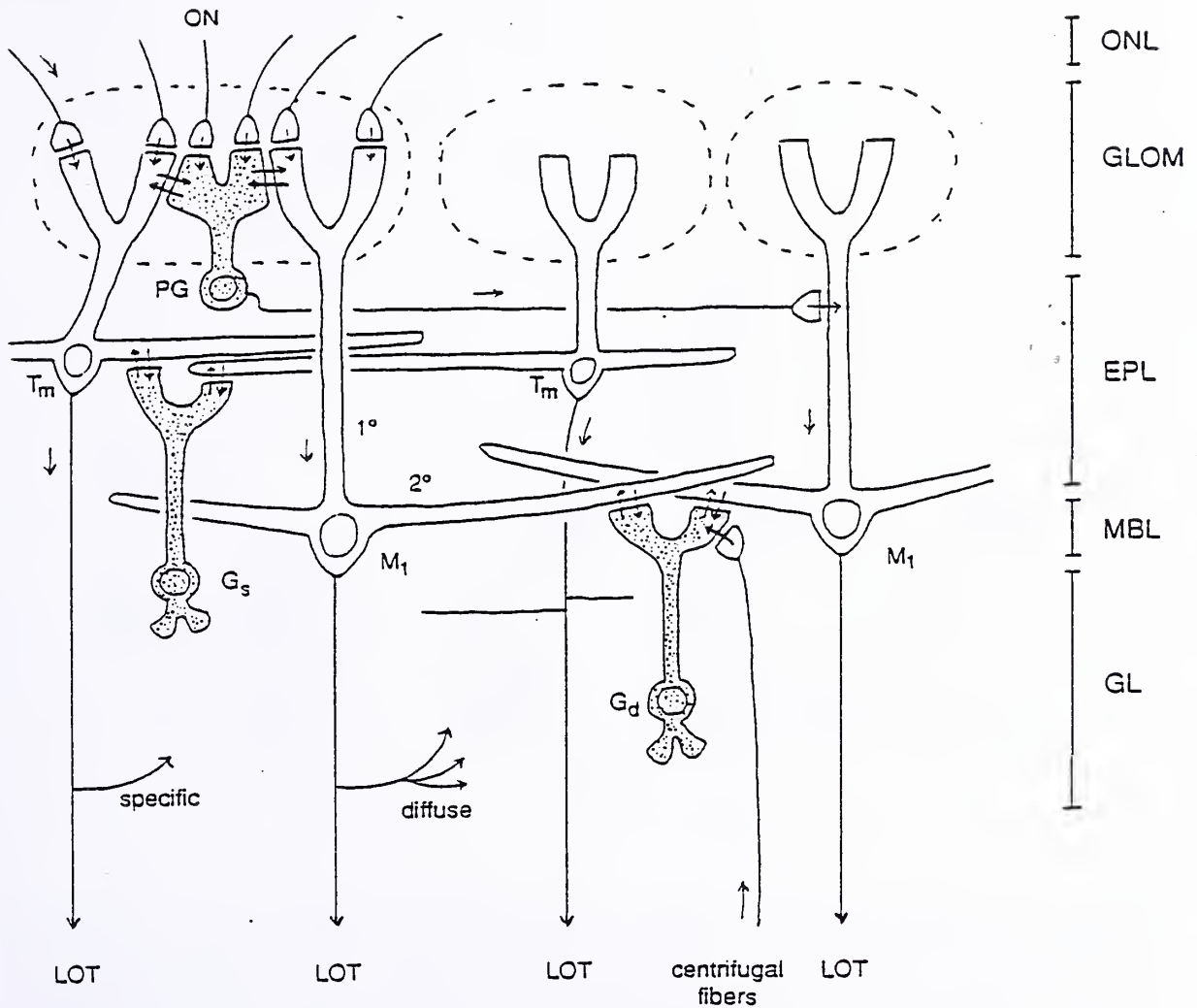


Figure 9. Basic circuit organization of olfactory bulb Abbreviations: ONL, olfactory nerve layer; GLOM, glomerular layer; EPL, external plexiform layer; MBL, mitral cell layer; GL, granule cell layer; ON, olfactory nerve fibers; M<sub>1</sub>, mitral cell subtype 1; T<sub>m</sub>, middle tufted cell; G<sub>s</sub>, superficial granule cell; G<sub>d</sub>, deep granule cell; LOT, lateral olfactory tract; PG, periglomerular; 1°, primary dendrite; 2°, secondary dendrite. Note the sublaminar distribution of tufted and mitral cell secondary dendrites and their potential connections with subpopulation of granule cells. (Adapted from Shepherd and Greer, 1990.)





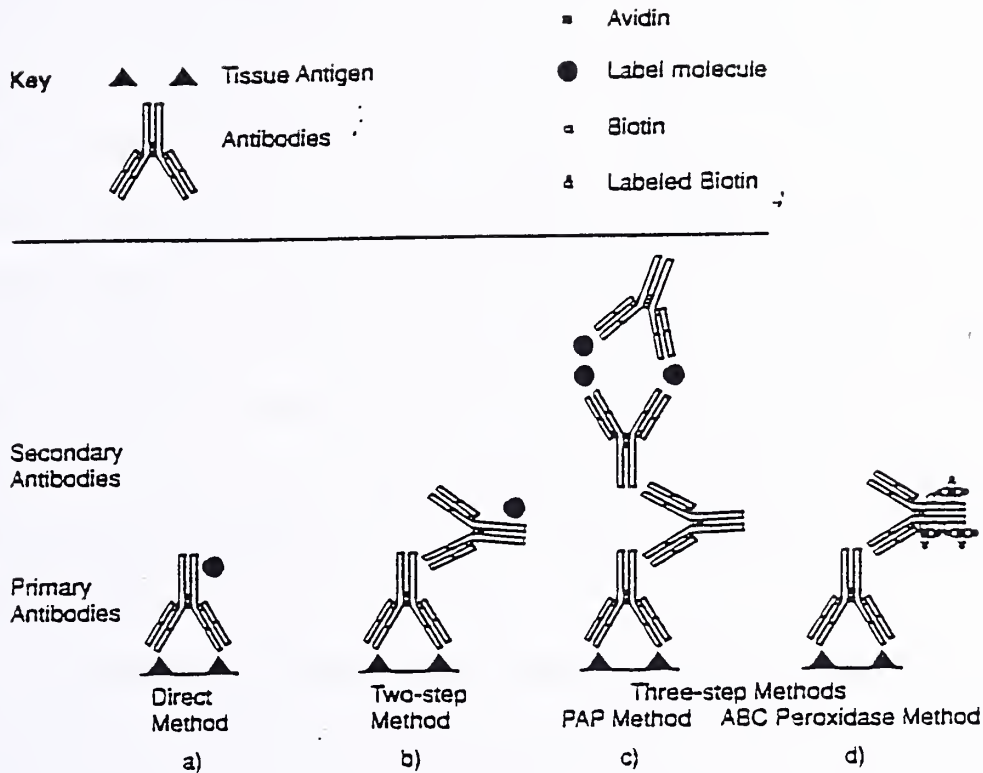


Figure 10. Four fundamental methods are the basis for most immunocytochemical techniques today: one-step (direct) methods (a), two-step methods (b), and three-step methods: PAP (c) and avidin-biotin techniques (d). Simplified diagrams are shown here indicating the basic binding sequences underlying each of these four methods. With the historical progression from the first methods to three-step methods that are so popular today, there have been tremendous increases in method specificity. The direct methods (a), where the primary antibody is itself labeled, are the least efficient techniques, largely because very few label molecules are conjugated to each antibody molecule. Two-step methods (b) are more efficient because several labeled secondary antibody molecules can bind to each molecule of primary antibody (not indicated in diagram), thereby resulting in more label bound to the tissue for each molecule of antigen. With the PAP (c) and avidin-biotin (d) methods, the third layer carries the label, and even greater efficiency is achieved for two major reasons: (1) several label molecules are bound in the PAP and avidin-biotin complexes, and (2) more than one secondary antibody can bind to each primary antibody, and, likewise, more than one PAP or avidin-biotin complex may bind to each secondary antibody. Therefore, with increasing layering of antibodies, increases in method efficiency are achieved. (Adapted from Beltz and Burd, 1989)



method. This method consists of a primary antibody labeled with several marker compounds that is directed to the antigen of choice. Available marker compounds include fluorescein, rhodamine, enzymes such as horse-radish peroxidase or glucose oxidase, or colloidal gold and ferritin particles. This method depends solely on the affinity of the antibody to the antigen. Therefore, through a conformational change the sensitivity of the antibody can be altered when trying to label the primary antibody with the marker compound. In order to obtain good staining very large quantities of antigen are needed. Another disadvantage to this method is the lack of flexibility in choosing a marker from one experiment to the next. Also, labeling every primary antibody is time consuming. The advantage of this method relies on the speed of achieving results. However, the disadvantages far outweighs the advantages and thus this technique is hardly used today.

The two step method relies on one antibody interacting with the antigen while a secondary antibody with the marker compound interacts with the primary antibody. The secondary antibody has to be directed against the IgG of the host animal in which the primary antibody was raised. Fluorescent markers such as rhodamine and fluorescein are often used in this technique for screening purposes. Enzymatic or peroxidase labeled compounds are employed for light and electron microscopy. The potential drawbacks of this method is that reduced specificity of the secondary antibody can result in non-specific binding leading to an increase in signal to noise ratio. The advantage of this method is on the increased sensitivity of interaction and visualization. Another advantage is the experimental flexibility offered in choosing different markers on the secondary antibodies.

In an attempt to increase the sensitivity of immunocytochemical methods, the three step method adds another level of secondary antibodies over the two step method. Today the most commonly used techniques employ the peroxidase-antiperoxidase (PAP) and the avidin-biotin complex (ABC). It is estimated that the PAP and ABC methods are



100 to 1000 times more sensitive than the two step method. The PAP molecules are pentagonal structures having a ratio of three molecules of peroxidase to two molecules of antiperoxidase. In the PAP technique (Figure 10C) the primary antibody binds to the antigen. The secondary antibody, raised in a different host from the primary, binds to the primary antibody but this time the antibody is not labeled. A tertiary antibody, raised in the same species as the primary, containing PAP molecules binds to the secondary antibody. The secondary antibody thus, act as a link between the primary and tertiary layer. Through an enzymatic reaction the PAP complex can be visualized on the tissue. The major advantage to this process is that more diluted concentrations of primary antibody can be used, thus, reducing background staining due to ionic interactions or low affinity antibodies in the serum. The major disadvantage is that the PAP molecule is large and can impair adequate penetration into tissue. By controlling the incubation periods, detergent concentration and temperature it is possible to overcome this obstacle (Sternberger et al., 1970).

Similar to the PAP method the ABC technique uses another immunoperoxidase reaction that by increasing the number of peroxidase molecules, further amplifies the staining signal. Biotin is a small vitamin molecule and avidin is a 68,000 molecular glycoprotein found in egg white and some bacteria. Avidin has a strong affinity for biotin and provides four active binding sites for biotin. In this technique the tissue is incubated in primary antibody followed by an incubation containing a biotin-labeled secondary antibody directed against the IgG of the host in which the primary antibody was raised. Multiple avidin molecules are covalently bound to this secondary antibody. The final step consists of an incubation period in a avidin-biotin peroxidase complex. The large number of peroxidase molecules and the high affinity of avidin to biotin allows for higher dilution of primary antibody. This reduces significantly the background with a high signal to noise ratio. The size of the complex reduces penetration into the tissue but as mentioned earlier, this can be partially overcome (Hsu et al., 1981). The added





advantage to the avidin molecule is that it can be covalently linked to other molecules such as fluorescent markers and colloidal gold. Colloidal gold particles can be used for light and electron microscopy. At the level of the electron microscope, gold particles of different size and raised in different hosts can be very useful in double label studies. In a similar vein, different fluorescent markers with varying activating light sources can be used for light microscopy in double labeling studies. In this study, the use of fluorescent markers and peroxidase staining were used for light and electron microscopy (respectively) observations of dopaminergic and GABAergic periglomerular cells (see below).

## 1. Dopamine

In the late 1950's dopamine was considered to be an intermediate in the synthesis of the noradrenaline. With improving histochemical techniques it was discovered that Parkinsonian patients had a substantial decrease of dopamine levels in the striatum. Subsequently, it was discovered that this decrease in dopamine was due to the loss of dopamine containing cells in the substantia nigra pars compacta, thus providing an insight in the role of dopamine in motor control (Hornykiewicz, 1966). In addition the recognition of antipsychotic drugs as antagonists of dopamine receptors provided another potential role for dopamine involved in thought process (Seeman et al., 1976). These discoveries have prompted an intense experimental effort in trying to understand the mechanism of dopamine as a neurotransmitter. The mesencephalon system where 80% of dopaminergic cells are located has been the area most studied in the brain (for a complete review refer to Lacey, 1989). These mesencephalic neurons are known to have extensive arborization and dopamine-containing vesicles. The postsynaptic membrane specializations are symmetrical and in the striatum, found predominantly on dendritic spines (Freund et al., 1984). This suggests that the role of dopamine, at least in the striatonigral pathways, is inhibitory.





However, there seems to be some controversy regarding the direct action of dopamine as an excitatory or inhibitory neurotransmitter, or as a more complex neuromodulator. Waszczak and Walters (1984) described a dopamine-GABA interaction in the substantia nigra pars reticulata where iontophoretically applied dopamine attenuated the inhibitory effect of GABA in the pars reticulata, thus modifying the output of neurons to the striatum. Adding to this complexity, dopamine-containing amacrine cells in the retina's inner plexiform layer have been shown to alter the size of the receptive field by uncoupling gap junctions between horizontal cells (Rogawski, 1987). Outside the central nervous system dopamine has been found to relax vascular smooth muscles (extensively studied in the kidneys) (Fredrickson et al., 1985) and inhibit the release of noradrenaline from sympathetic nerves (Bell, 1988).

There are at least six major types of dopamine receptors. All of these are known to act on G proteins in various ways. The two most thoroughly studied dopamine receptors are D<sub>1</sub> and D<sub>2</sub>. The D<sub>1</sub> receptors stimulate the production of cAMP by activating adenylyl cyclase. D<sub>1</sub> receptors have been identified in neurons postsynaptic to dopamine containing afferents (Lacey, 1989). Interestingly, in the striatum, D<sub>1</sub> receptors have been found on GABA-containing neurons which compromise about 95% of the total neuronal population (Palacios and Pazos, 1987). This observation adds to the possible interaction between GABA and dopamine. D<sub>2</sub> receptors are known to reduce cAMP levels (Kebabian, and Calne, 1979). These receptors are usually located presynaptically to dopamine-containing cell bodies and dendrites. Activation of these receptors leads to inhibition of dopamine production by inhibition of tyrosine hydroxylase (Roth, 1984). A plausible explanation of this phenomenon might lie in the inhibition of cAMP through the D<sub>2</sub> receptors which then inhibit the cAMP-dependent phosphorylation that activates tyrosine hydroxylase (Lacey, 1989).

As mentioned previously, dopamine-containing neurons in the olfactory bulb are mostly confined to the glomerular layer (Halász et al., 1981a, 1982, 1983, 1985).



Immunocytochemical studies employing antibodies against the rate limiting enzyme for dopamine (tyrosine hydroxylase) demonstrated reactivity within the glomerulus against tyrosine hydroxylase but not against dopamine- $\beta$ -hydroxylase. This confirms the notion that periglomerular cells are capable of converting dopa to dopamine, yet unable to convert dopamine into norepinephrine (Hökfelt et al., 1985; Halász, 1977). In order to establish if the dopamine was endogenous to the olfactory bulb several olfactory nerve deafferentation studies have shown that dopamine is not utilized by the olfactory nerve as a neurotransmitter. Importantly, these studies showed a reduction of dopamine and tyrosine hydroxylase expression in the glomerular layer (Margolis et al., 1974; Keller and Margolis, 1975; Nadi et al., 1981; Baker, 1983; Stone et al., 1991). This reduction was not due to cell degeneration but possibly secondary to loss of neuronal activity or loss of a trophic substance by the afferent fibers during synaptic transmission (Baker et al. 1988). This type of transsynaptic control of tyrosine hydroxylase activity has been well documented in other areas such as the adrenal medulla (Guidotti and Costa, 1977) carotid body and superior cervical ganglion (Zigmond and Chalazonits, 1977), and striatum (Roth et al., 1974). After deafferentation this reduction of tyrosine hydroxylase activity was followed by an increase in activity subsequent to the regeneration and reinnervation of olfactory receptor cell axons terminals. This observation implies that the transsynaptic control is regulated by the olfactory receptor cell axons (Margolis et al., 1974; Nadi et al., 1981; Baker, 1983).

Centrifugal fibers from the nucleus of the lateral olfactory tract and diagonal band fibers appear to be another source of dopamine in the olfactory bulb (Brownstein et al. 1974; Veersteeg et al. 1976). Central dopaminergic projections from the ventral tegmentum and substantia nigra were identified as terminating intensely in the internal plexiform layer and less intensely in the periglomerular regions (Fallon and Moore, 1978). However, Macrides et al. (1981) were not able to find such centrifugal afferents in the hamster. From the aforementioned and several additional experiments (see Halász



and Shepherd, 1983 for a comprehensive review) it is highly likely that the majority of dopamine-containing neurons are located mainly around the glomeruli specifically within the intrinsic, periglomerular, superficial tufted, and short axon neurons. This observation is supported by biochemical, histochemical and lesion studies (Halász and Shepherd, 1983).

The role of dopamine in the olfactory bulb remains controversial. Utilizing dopamine receptor agonists and antagonists Nowycky et al. (1983) studied the effects of exogenous dopamine in the olfactory bulb. They showed that dopamine agonists reduced the amplitude of the potential in mitral cells evoked by orthodromic or antidromic stimulation, while the dopamine antagonist increased the response. This led to the conclusion that endogenous dopamine found in the periglomerular cells might serve as an inhibitor to mitral cells. In a recent study, (Nickell et al., 1991) high levels of the dopamine D<sub>2</sub> receptor were found in the olfactory nerve layer while D<sub>1</sub> receptors were homogeneously distributed at lower concentrations throughout the bulb. This led to a new potential role for dopamine as neuroregulator of neurotransmitter release from olfactory nerve terminals. In addition, it is well accepted that dopamine and GABA colocalize in periglomerular cells (Kosaka et al. 1985; Gall et al. 1987). The combination of two inhibiting neurotransmitters raises new questions regarding the function of dopamine and GABA. As explained by Gall et al. (1987), maybe there is a synergistic effect from these neurotransmitters via different mechanisms. GABA is known to inhibit postsynaptic targets by opening chloride channels (Alger and Nicoll, 1983). Dopamine is believed to hyperpolarize neurons and to prolong hyperpolarization via a calcium dependent potassium channel (Bernardo and Price, 1982). As will be explained later in the Current Study section, the extent to which receptor cell axons distribute among the subpopulation of periglomerular cells continues to be unknown and this information could shed some insights into understanding the role of dopamine and GABA in the glomerular layer.





## 2. GABA

Since the 1960's it was established that GABA and glycine acted as inhibitory neurotransmitters in the nervous system. GABA is extensively distributed in the central nervous system and found in high concentrations in the hypothalamus, hippocampus and basal ganglia of the brain and in the substantia gelatinosa of the spinal cord and in the retina. Two types of receptors have been identified for GABA, GABA<sub>A</sub> and GABA<sub>B</sub>. GABA<sub>A</sub> receptors are those sensitive to the antagonists actions of biculline and picrotoxin. GABA<sub>B</sub> are insensitive to biculline and picrotoxin but baclofen acts as an agonist.

GABA<sub>A</sub> receptors are predominantly found in neurons and glial cells and are believed to be present in postsynaptic targets, thus mediating the classical inhibitory effects of GABA. The structural and amino acid composition of GABA<sub>A</sub> receptors is beyond the scope of this thesis but it is sufficient to say that these are composed of several subunits, mainly  $\alpha$ ,  $\beta$ ,  $\delta$ , and  $\gamma$  (Stephenson and Dolphin, 1989). Each of the  $\alpha$ , and  $\beta$  isoform subunits transcripts have a specific regional domain within the brain demonstrated by *in situ* hybridization and Northern blots (Levitan et al., 1988; Wisden et al., 1988). As an example, the  $\alpha 1$  mRNA is the most abundant in the cerebral cortex while the  $\alpha 2$  mRNA is found in high concentrations in the hippocampus. The apparent heterogeneity of these receptors allows for distinct allosteric modulatory sites for benzodiazepines, barbiturates and some steroids which potentiate the effect of a GABA<sub>A</sub> receptor response. Although the electrophysiological function of GABA<sub>A</sub> receptors is, again, beyond the scope of this thesis (refer to Bormann for complete description, 1988) it is sufficient to say that the main inhibitory action of GABA<sub>A</sub> is as a consequence of increasing cell membrane conductance to chloride.

The less well studied GABA<sub>B</sub> receptors are presynaptic and found predominantly in dendrites in the molecular layer of the cerebellum, the frontal cortex and the substantia





gelatinosa of the dorsal horn of the spinal cord (Stephenson and Dolphin, 1989). The inhibitory action of GABA on GABA<sub>B</sub> receptors results from an inward potassium current with subsequent membrane hyperpolarization with decreasing input resistance. In addition, they appear to inhibit voltage activated calcium channels via a pertussis-toxin-sensitive G protein (Bowerly, 1989). The functional significance of two mechanisms of GABA for inhibitory control remains enigmatic. It is known that most GABA-containing neurons are local interneurons that regulate neurotransmission. Some clinical diseases can be treated by drugs that interact with GABA receptors. Such diseases include epilepsy where the GABA<sub>A</sub> antagonist bicuculline can induce seizures and spasticity which can be somewhat relieved with baclofen. Another well studied disease is anxiety disorder where GABA<sub>A</sub> agonists relieve the symptoms (Bowerly and Nistico, 1989).

Several subpopulation of GABA-containing neurons have been identified in the granule and periglomerular layer of the olfactory bulb (Halász et al., 1979, 1981b; Halász and Shepherd, 1983; Kosaka et al., 1987). After performing deafferentation studies there was no evidence to suggest the presence of GABAergic centrifugal or central projections into the olfactory bulb since there were no changes in exogenous GABA uptake (Margolis, 1974). Similarly Harvey et al. (1975) did not find any changes in GABA levels in the guinea-pig olfactory cortex under similar conditions, suggesting that there is no GABAergic afferent pathway from the olfactory bulb into the cortex. Therefore, GABAergic containing neurons appear to be mostly intrinsic to the olfactory bulb. Immunocytochemical studies have shown that the highest concentration of GABA and GABA related compounds are found in granule cell bodies and dendrites. At the level of the light and electron microscope, glutamic acid decarboxylase (rate limiting enzyme for GABA synthesis) (GAD) reactive processes were identified as belonging to periglomerular and granule cells. The GAD-reactive granule cell dendritic spines were seen establishing reciprocal dendrodendritic synapses with mitral/tufted cell secondary dendrites located in the external plexiform layer (Ribak et al. 1977, 1981; Rall et al.,



1966). In addition several GABA reactive axonal boutons were identified as those potentially belonging to short axon cells establishing synaptic contact with granule cell dendrites and somata in the granule cell layer. Other reactive axons were seen in the glomerular and external plexiform layer (Halász et al., 1979).

There exists little doubt that GABA exerts an inhibitory action in the olfactory bulb. This is supported by studies employing iontophoresis of GABA on olfactory bulbs on cats (Nicoli, 1970, 1971) and rabbits (MacLennan, 1971; Felix and MacLennan, 1971). After olfactory nerve stimulation the mitral cells were hyperpolarized, presumably by GABA since this action was reversed with the application of biculline or picrotoxin. A similar response was obtained by antidromic stimulation of the lateral olfactory tract. This led to the conclusion that granule cells through reciprocal synapses exert their GABAergic inhibitory action on mitral/tufted cells within the external plexiform layer. Studies on turtle olfactory bulbs showed that after orthodromic and antidromic stimulation the intracellular response of mitral cells included a sequence of hyperpolarization. These responses also are most likely mediated by GABA since they were blocked by biculline and low concentrations of chloride into the bathing medium (Mori and Shepherd, 1979; Waldow et al., 1981). The majority of GABA receptors in the external plexiform layer are of the GABA<sub>A</sub> subtype while there seems to be an equal density of both GABA<sub>A</sub> and GABA<sub>B</sub> subtype in the glomerular layer (Bowery et al., 1987), although the significance of this differentiated distribution is not understood.

It is believed that analogous to the reciprocal synapses found in the external plexiform layer, GABA immunoreactive periglomerular cells exert an inhibitory action on mitral/tufted cell apical dendrites within a glomerulus. However, the coexistence of dopamine in these neurons raises new questions regarding the role of GABA. Perhaps during certain physiological situations the different mechanisms and durations of action of dopamine and GABA (see above) might cause one neurotransmitter to dominate over the other neurotransmitter. The short acting inhibition of GABA will dominate when



there is a short stimulus arising from the olfactory nerve. However, when the stimulus is prolonged or repeated, activation of periglomerular cells might activate the dopamine circuit causing a more prolonged hyperpolarization of mitral/tufted cells (Gall et al., 1987).

#### **D. SIGNIFICANCE OF OLFACTORY BULB IN NEUROSCIENCE RESEARCH**

From a historical perspective the organization of the olfactory bulb aided Cajal and other scientists in the understanding and acceptance of the neuron doctrine. Blanes (1890) argued Golgi's theory of reticularism which declared that axons were responsible for signal transmission. Golgi stated that the axons of olfactory receptor cells transmitted the odor signal onto other axons located within the glomerulus and that the dendrites only had a trophic function. By studying the olfactory bulb laminar organization, Blanes proposed that it was futile to have millions of olfactory receptor cell axons converging into a single glomeruli without making some form of contact with the mitral/tufted cell dendrites, as Golgi stated. Therefore, Blanes and Cajal concluded that odor signals arising from the nose are transmitted into the dendrites of tufted and mitral cells in the glomerulus, and escape the olfactory bulb through their axons.

Today the olfactory system is recognized as an optimal model for understanding principles of neuronal organization. Moreover, the olfactory system is one of two systems in the CNS to have continual regeneration of receptor cell axons (Graziadei, 1973; Graziadei and Monti-Graziadei, 1983). Each receptor cell axon originating from the olfactory epithelium survives for approximately 60 to 90 days when they undergo programmed cell death. The concept of apoptosis has attracted the attention of many scientists especially in the field of oncology, in trying to understand the molecular and genetic regulation inherent in cell death. Cloning the gene responsible for cell death could bring many therapeutic modalities, such as a gene novel therapy directed against cancer. Of particular interest in our laboratory is that once a receptor cell axon





disappears, a new regenerating axon establishes specific synaptic contact within the glomeruli via unknown neuroregulatory mechanisms. Several studies (Graziadei and Kaplan, 1980) have transplanted occipital cortex after bulbectomies and have found that olfactory nerve axons regenerated and formed characteristic glomerular structures in the transplanted cortex. This suggests an intrinsic capacity of olfactory axons to reorganize and establish synaptic contact. However, in a mutant strain of mice White (1973) noted that the asymmetrical synapses between the olfactory axon and periglomerular cells were absent. This observation raises two possible explanations. First, that a specific signal from the periglomerular cells controlling the olfactory axons termination is deficient. Alternatively, that there exists a subpopulation of olfactory axon arising from the epithelium that establishes contact specifically with periglomerular cells and that this subpopulation of receptor cells is missing in this BALB/c mice. The former seems to be the most plausible explanation since studies have revealed that the olfactory axons are organized into tight fascicles proximal to the olfactory epithelium and these desegregate when they reach the glomerular layer and target specific glomeruli (Greer and Kaliszewski, 1992). Thus, this evidence suggests the possibility that within individual glomeruli there are specific extrinsic cues controlling the termination of olfactory axons.

There are many pathological diseases that alter the anatomical structure of the olfactory bulbs. Approximately five percent of head trauma is associated with loss of smell secondary to axonal shear at the cribriform plate. Unfortunately, only 15 to 39 percent of patients recover the sense of smell (Sumner, 1964; Zusho, 1982; Costanzo and Becker, 1986). In addition, increasing age has been documented to reduce olfactory thresholds, possibly related to olfactory bulb atrophy (Doty et al., 1984; Cain et al., 1988). Disorders of smell have been associated with a number of conditions including endocrine, neurological, psychiatric and nutritional disorders (refer to Table 1). A well documented endocrine disorder that has captured the attention of neuroscientists is





Table 1. Medical Conditions Affecting Smell. Adapted from Schiffman (1983)*Nervous*

Alzheimer's disease  
 Down's Syndrome  
 Epilepsy  
 Head Trauma  
 Korsakoff's syndrome  
 Migraine  
 Multiple Sclerosis  
 Parkinson's disease  
 Pick's disease  
 Tumors and lesions

*Nutritional*

Chronic renal failure  
 Liver diseases including cirrhosis  
 Vitamin B12 deficiency

*Endocrine*

Adrenal cortical insufficiency  
 Cushing's syndrome  
 Hypothyroidism  
 Diabetes mellitus  
 Turner's syndrome  
 Kallman's syndrome  
 Primary amenorrhea  
 Pseudohypoparathyroidism  
 X-linked ichthyosis due to steroid sulfatase deficiency

*Local*

Adenoid hypertrophy  
 Allergic rhinitis, atrophy, and  
     bronchial asthma  
 Crouzon's syndrome  
 Leprosy  
 Ozena  
 Paranasal sinuis exenteration  
 Sinusitis and polyposis  
 Sjögren's syndrome

*Viral and Infectious*

Acute viral hepatitis  
 Influenza like infections

*Other*

Amyloidosis and sarcoidosis  
 Familial (genetics)  
 Laryngectomy  
 Psychiatric disorders



Kallman's syndrome (Kallman et al., 1944). In Kallman's syndrome, patients have hypogonadotropic hypogonadism with associated congenital anosmia secondary to aplasia or hypoplasia of the olfactory bulb and tracts (DeMorsier, 1954). During development the Gn-RH-secreting neurons and olfactory neurons originate from the olfactory placode, the precursor of the olfactory bulbs. Both olfactory axons and Gn-RH share a common migration pathway, but Gn-RH neurons migrate along the olfactory nerves to eventually reach their final destination in the hypothalamus. Since axonal and cell migration are likely to be dependent on guidance molecules, it is believed that patients with Kallman's syndrome are deficient in expressing these necessary proteins (Schwanzel-Fukuda, et al., 1989). Today the gene responsible for producing the *Kal* protein has been identified and further work is currently seeking to characterize the pathogenesis of Kallman's syndrome (Rugarli and Ballabio, 1993). Other endocrine disorders associated with smell impairment are women with primary amenorrhea (Marshall and Henkin, 1971), patients with Turner's (Henkin, 1976) and Cushing's syndrome (Kallman et al., 1944) to name a few.

Olfactory dysfunction are sometimes observed in patients with neurologic impairment. Patients with demyelinating diseases such as multiple sclerosis have been shown to express deficits in smell, depending on the distribution of disease (Pinching, 1977; Catalanotto et al., 1986). Patients with temporal lobe epilepsy are slightly deficient in odor identification and recognition. These deficiencies exacerbate after temporal lobectomy with greater impairment for the nostril ipsilateral to the lesion (Eskenazi, 1986). Parkinson's and Alzheimer's diseased patients show deficits in odor identification and thresholds (Ansari and Johnson, 1975; Doty et al., 1987; Ohm, 1987). Alzheimer's patients were also found to have phosphorylated neurofilaments in the olfactory epithelium (Talamo et al., 1989). This finding could potentially result in olfactory epithelium biopsies as a means to diagnose this disease. Also found in Alzheimer's patients are neuritic plaques and neurofibrillary tangles segregated throughout olfactory



centers in the brain including the uncus, anterior olfactory nucleus and amygdaloid complex, all of which receive direct connections from the olfactory bulb. Other dementia related disorders known to affect olfactory centers are Huntington's chorea (Moberg et al., 1984), and Korsakoff's psychosis Jones et al., 1975; Mair et al., 1986). These observations have raised the possibility that if an infectious agent exists for this diseases then the olfactory system could be the initial site and possible source of route (Pearson et al., 1985; Shipley, 1985).

## **E. CURRENT STUDY**

At this time I would like to thank Christine Kaliszewski for her endless patience in teaching me the immunocytochemical and electron microscopic techniques employed during the three studies discussed below. All of the techniques were completed by the medical student except for those otherwise noted.

Using the information presented above three studies will attempt to examine the anatomical heterogeneity and synaptic organization of the rat olfactory bulb glomerulus. In the first study the segregation of subpopulations of periglomerular cells immunoreactive to tyrosine hydroxylase and GABA will be examined using the light microscope. The second study will employ transglomerular montages at the level of the electron microscope to characterize the synaptic organization and elucidate discrete anatomical patterns of connectivity for an entire glomerulus. The third study will attempt to characterize the synaptic organization of tyrosine hydroxylase and GABA immunoreactive processes at the level of the electron microscope. It is evident that these findings will serve as a model for understanding mechanisms of organization of local synaptic circuits elsewhere in the central nervous system. It will also shed some insights into the etiology of diseases affecting the olfactory system.

This is the first study to approach glomerular organization with the intent of understanding the degree to which synaptic circuits are heterogeneously distributed





within distinct subglomerular domains. Moreover, this is the first study to quantitatively approach the crucial question of whether the distribution of synaptic circuits is equivalent among identified subpopulations of glomerular layer interneurons. Ultimately, it is hoped that this information will bear upon the current controversy of how different odors or components of odor molecules may be processed through parallel pathways in the olfactory system.



## **II. STUDY I-LIGHT MICROSCOPY**

### **A. PURPOSE OF STUDY**

As mentioned previously, periglomerular cells can be divided based on their immunohistochemical characterization. Subpopulations of these neurons are immunoreactive to GABA, tyrosine hydroxylase and others colocalize both neurotransmitters. This study will attempt to describe the distribution of periglomerular cells immunoreactive to GABA and tyrosine hydroxylase employing fluorescent microscopy. The differential distribution of these neurotransmitters might imply a heterogeneous synaptic organization of local circuits in the olfactory glomerulus. This proposed heterogeneity of synaptic organization for periglomerular cells immunoreactive for GABA and tyrosine hydroxylase will be studied further in Study III.

### **B. TISSUE COLLECTION**

Four adult Sprague Dawley rats (3 males, 1 female) weighing 170- 280 grams were euthanized with approximately 90mg/Kg of sodium pentobarbital (Nembutal) injected into the peritoneal cavity. The absence of corneal reflex was used to determine the effectiveness of anesthesia. The chest was opened and the heart exposed. A small incision was made in the right atrium and the rats were perfused via the left ventricle with 0.9% normal saline and 1% heparin (approximately 1000U/ 50ml saline) until the liver blanched and the extremities became pale. Following the infusion of saline , the rats were perfused with 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1M phosphate buffer. Approximately 200 to 300ml was given over a 30 minute period. The brain was exposed and removed with careful attention to removal of the meninges. The brains were placed in the perfusate solution of 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1M phosphate buffer on ice for 1 to 2 hours of post-fixation.



Following post-fixation the brains were washed in 0.1M phosphate buffer (PB), pH 7.4 2X for 15 minutes and placed onto a block with cyanoacrylate (Krazy Glue) and embedded in agar. Once the agar hardened, the bulbs were sectioned with a Pelco 101 vibratome at a speed of 4, amplitude of 5, and a blade angle of 20 degrees into 50µm thick coronally sliced sections. Sections that were to be stained the same day were placed in a tissue culture wells filled with 0.1M PB. The other sections were placed in a petri-dish filled with anti-freeze solution and stored at -20°C until needed. The anti-freeze solution was made by combining the following substances: 500ml of 0.1M PB; 300 grams of sucrose; 10 grams of polyvinylpyrrolidone; 300ml ethylene glycol; and water. At the end of 7 days unused frozen tissue was discarded..

### C. STAINING PROCEDURE

Free floating sections were counter stained for GABA and tyrosine hydroxylase immunofluorescence through the following technique.

- 1) Between five to eight sections were placed on each well. Each well was rinsed five to 6X 10 minutes with 0.1M PB.
- 2) Washed in a pretreatment solution composed of 3% normal serum ( normal goat serum for tyrosine hydroxylase and normal rabbit serum for GABA) in 0.1M PB for 60 minutes to block the non-specific binding sites.
- 3) Following pretreatment the sections were rinsed in 0.1M PB 2X 10 minutes .
- 4) After rinsing the sections were placed in test tubes and incubated for 48 hours at a temperature of 4°C in the primary antibody for GABA and tyrosine hydroxylase raised in guinea pig and rabbit, respectively (Eugene Tech). The concentrations for the primary antibodies were 1:400 for both, GABA and tyrosine hydroxylase. The sections were constantly agitated during the incubation period.
- 5) Following incubation, the sections were washed 5X 10 minutes each in 0.1M of PB. The sections incubated with anti-tyrosine hydroxylase antibody were then



incubated in anti-rabbit Texas Red labeled IgG (Vector Labs) at a dilution of 1:200 for 1 hour. The sections incubated in anti-GABA antibody were incubated for 1 hour in biotinalated anti-Guinea Pig IgG (Vector Labs) made in goat.

6) Following incubation in the secondary antibodies, the sections were washed 3X 10 minutes each in 0.1M PB. The GABA labeled sections underwent another incubation period with avidin fluorescein labeled molecules (Vector Labs) at a dilution of 1:200. Following incubation the sections stained for GABA underwent three washes ten minutes each in 0.1M PB. A section from each well was then mounted on a slide and visualized under the fluorescent microscope for evidence of staining.

7) Once adequate staining was established, the sections immunoreactive to tyrosine hydroxylase were placed in test tubes and incubated in primary guinea pig anti-GABA antibodies for 48 hours at 4°C and a concentration of 1:400. Those sections immunoreactive to GABA were placed in test tubes and incubated for 48 hours at 4°C in primary rabbit anti-tyrosine hydroxylase antibodies at a dilution of 1:400.

8) Repeated procedure 5 and 6.

9) The sections were placed on subbed slides and a coverslipped with Vectashield (Vector Labs) which reduces fading of the immunofluorescent markers.

10) Two tissue sections were selected and stained with 0.01% ethidium bromide after acquiring the necessary data. Ethidium bromide nonselectively stains cellular DNA.

11) Control: To determine method specificity, tissue sections were put through the staining procedure without primary antibody. These sections showed no specific staining pattern.

#### **D. DATA AQUISITION AND ANALYSIS**

The sections were visualized and studied under an Olympus epifluorescent microscope equipped with rhodamine and FITC filters. The slides were examined under low power light microscopy (40X magnification using an 4X objective). Colocalization





studies were performed by alternating the fluorescent filters under the same tissue field. The cell counts presented below represent pooled total counts of all immunoreactive neurons within four olfactory bulbs from two animals.

Figures presented in this section were acquired from a BioRad 600 confocal microscope. By exchanging laser filters the activation wavelengths for Texas Red and fluorescein were obtained. In an attempt to acquire unequivocal immunoreactive cell profiles, special attention was taken to balance the gain of the laser beam for both the fluorescein and Texas Red filters. The images were acquired and stored on a Elegance 433i Northgate computer and photographs from the monitor were taken with a Nikon N6006 camera. The colors illustrated in the figures are computer reconstructed, so that green stained neurons are GABA immunoreactive, red stained neurons are tyrosine hydroxylase immunoreactive, and yellow neurons represent colocalization. Figures stained with blue are representative of all neurons within the field; these neurons were stained with 0.01% ethidium bromide.

## **E. RESULTS**

### **1. Normal Olfactory Bulb Histology**

For comparison purposes, the normal laminar organization of the olfactory bulb is shown in figure 6. This represents a 10 $\mu$ m thick section stained with cresyl violet. Cresyl violet stains selectively neuronal cell bodies and does not stain neuronal processes.

The five layers of the olfactory bulb are well illustrated in figure 6. The most superficial layer is the olfactory nerve layer with scattered cell bodies most likely glial cells. Immediately below the olfactory nerve layer is the glomerular layer with their distinctive cylinder like structures. Individual glomeruli are well delineated by the high density of small periglomerular cells. Deep to the glomerular layer is the external plexiform layer where the cell bodies of the three subtypes of tufted cells are located.



The external plexiform layer is mostly comprised of the dendritic processes from the mitral, tufted and granule cell (see above). Immediately adjacent to the external plexiform layer lies the mitral cell layer where the large mitral cell bodies reside. The deepest layer is the granule cell layer with its characteristic clusters of granule cell bodies seen throughout the entire layer.

## 2. GABA and Tyrosine Hydroxylase

Figure 11 shows a low magnification of the olfactory bulb stained for GABA (A) and tyrosine hydroxylase (B). Notice the numerous cell bodies immunofluorescent for GABA (arrows) present in the glomerular layer surrounding individual glomeruli (GLOM in Figure 11A). A few stained cell bodies are also found in the external plexiform layer (EPL in Figure 11A) and a higher density of stained cells are found deeper in the granule cell layer (GRL in Figure 11A). Tyrosine hydroxylase cell bodies (arrows) (Figure 11 B) are found in high concentration surrounding individual glomeruli but in contrast to GABAergic cells there is a lower density in the external plexiform layer and almost none are seen in the granule cell layer. Figure 13 represents another low magnification of the olfactory bulb demonstrating the laminar distribution for GABA (A) and tyrosine hydroxylase (B) immunoreactive cell bodies. The GABA immunoreactive cell bodies are found in higher concentration in the glomerular and granule cell layers. The concentration of tyrosine hydroxylase immunoreactive cell bodies (arrows) decreases from the glomerular layer deeper into the granule cell layer. Among the tyrosine hydroxylase immunoreactive cell bodies there were larger cells (arrow heads) usually located in the glomerular-external plexiform layer border. Based on their morphology and location these cells were identified as superficial tufted cells (Pinching and Powell, 1971a,b; Schneider and Macrides, 1978).





Figure 11. Low magnification of rat olfactory bulb immunohistochemically stained using anti-GABA (A) and anti-tyrosine hydroxylase (B) primary antibodies. The fluorescent marker for GABA was fluorescein and for tyrosine hydroxylase, Texas Red. The color represented in these pictures are computer generated to facilitate interpretation of immunoreactivities. Abbreviations: ONL, olfactory nerve layer; GL, glomerular layer; EPL, external plexiform layer; GRL, granule cell layer; GLOM, glomerulus. Arrows indicate individual and identical immunoreactive cell bodies.









Figure 12. Superimposed image of figures 11 A and B demonstrating colocalization of GABA and tyrosine hydroxylase by yellow colored cell bodies (arrows). Notice immunoreactive cell bodies (green colored) for GABA found in higher density in the granule cell layer. Abbreviations: ONL, olfactory nerve layer; GL, glomerular layer; EPL, external plexiform layer; GRL, granule cell layer; GLOM, glomerulus. Arrows indicate individual immunoreactive cell bodies. The reader should compare and recognize that these cells are identical to those from figure 11 A and B. Scale bar= 100 $\mu$ m.









Figure 13. Low magnification of rat olfactory bulb immunohistochemically stained using anti-GABA (A) and anti-tyrosine hydroxylase (B) primary antibodies. Notice laminar distribution of GABA and tyrosine hydroxylase immunoreactive cell bodies. GABA immunoreactive cells are present in high density in the glomerular and granule cell layers. Tyrosine hydroxylase immunoreactive cell bodies are located in the glomerular region and to a lesser extent in the external plexiform layer. Superficial tufted cell bodies (arrow head) are located in the glomerular-external plexiform layer border and are exclusively immunoreactive to tyrosine hydroxylase. Also notice individual periglomerular cell immunoreactive for GABA only (open arrow head). Abbreviations: ONL, olfactory nerve layer; GL, glomerular layer; EPL, external plexiform layer; GRL, granule cell layer; GLOM, glomerulus. Arrows indicate individual and identical immunoreactive cell bodies.

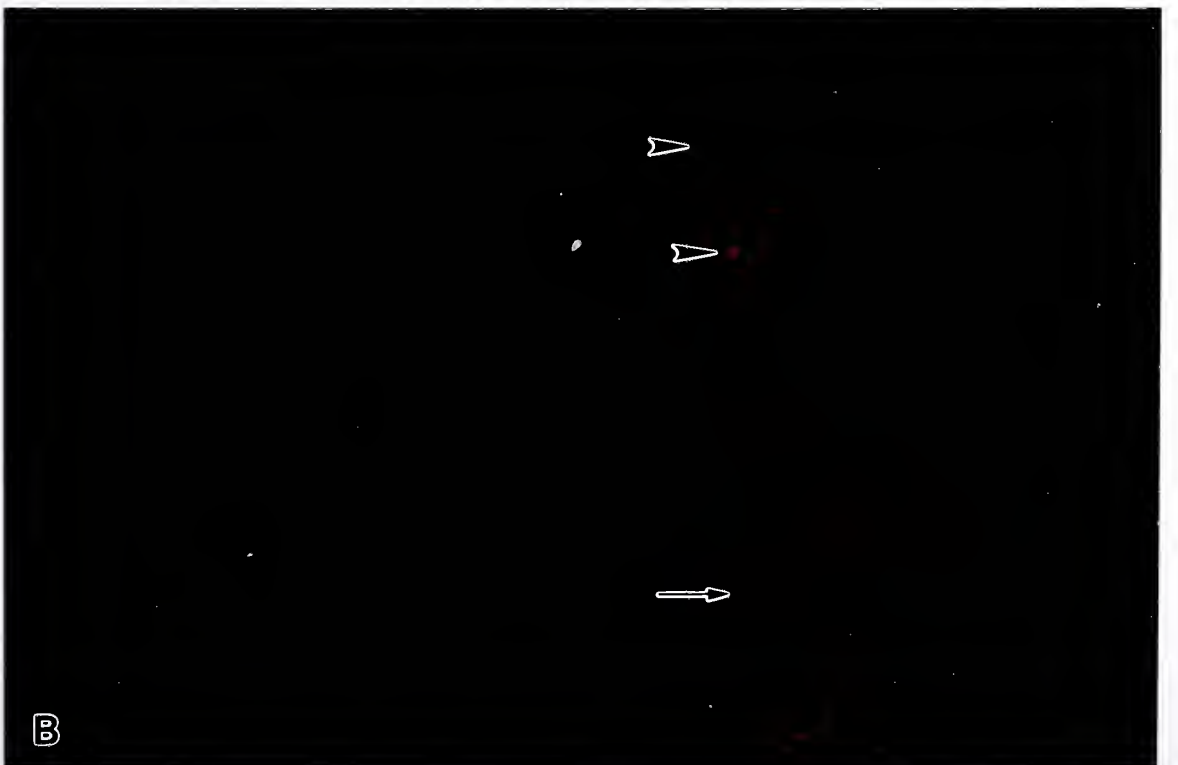
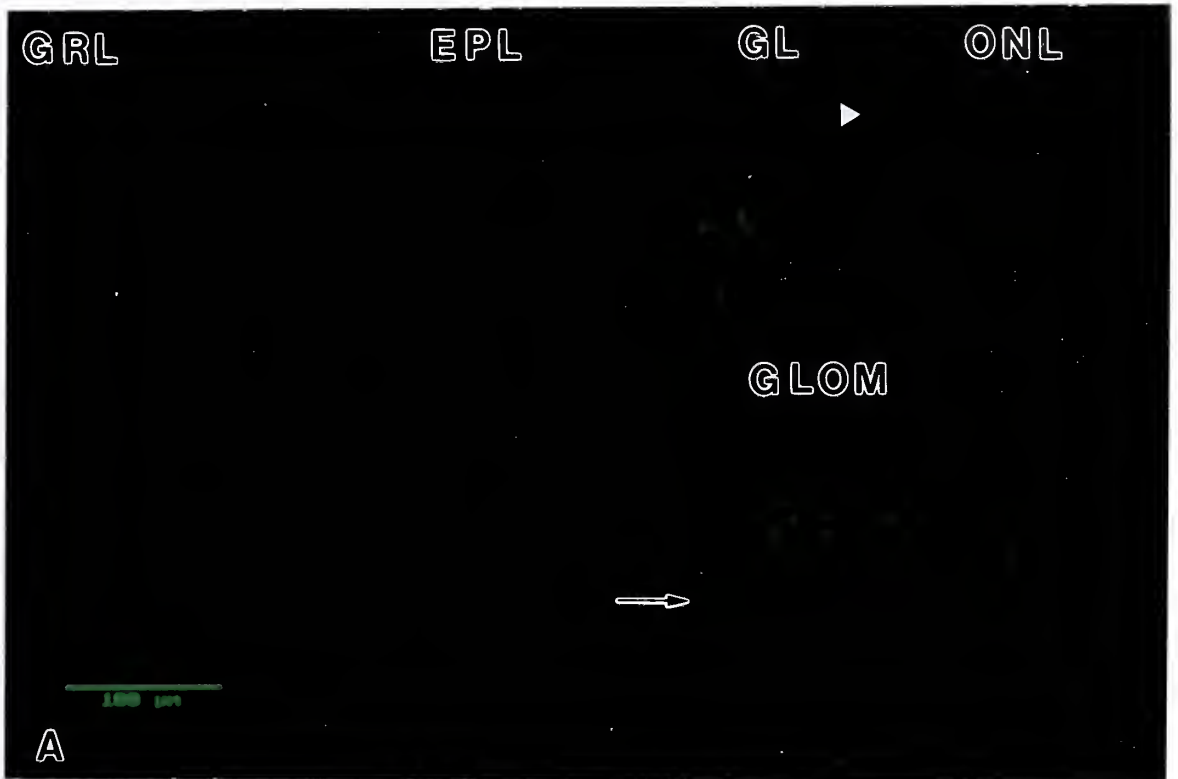






Figure 14. Superimposed image of figures 13 A and B demonstrating colocalization of GABA and tyrosine hydroxylase by yellow colored cell bodies (arrows). Superficial tufted cells are identified by their immunoreactivity to tyrosine hydroxylase (arrow head). GABA immunoreactive periglomerular cell is shown (white arrow head). Abbreviations: ONL, olfactory nerve layer; GL, glomerular layer; EPL, external plexiform layer; GRL, granule cell layer; GLOM, glomerulus. Scale bar= 100 $\mu$ m.

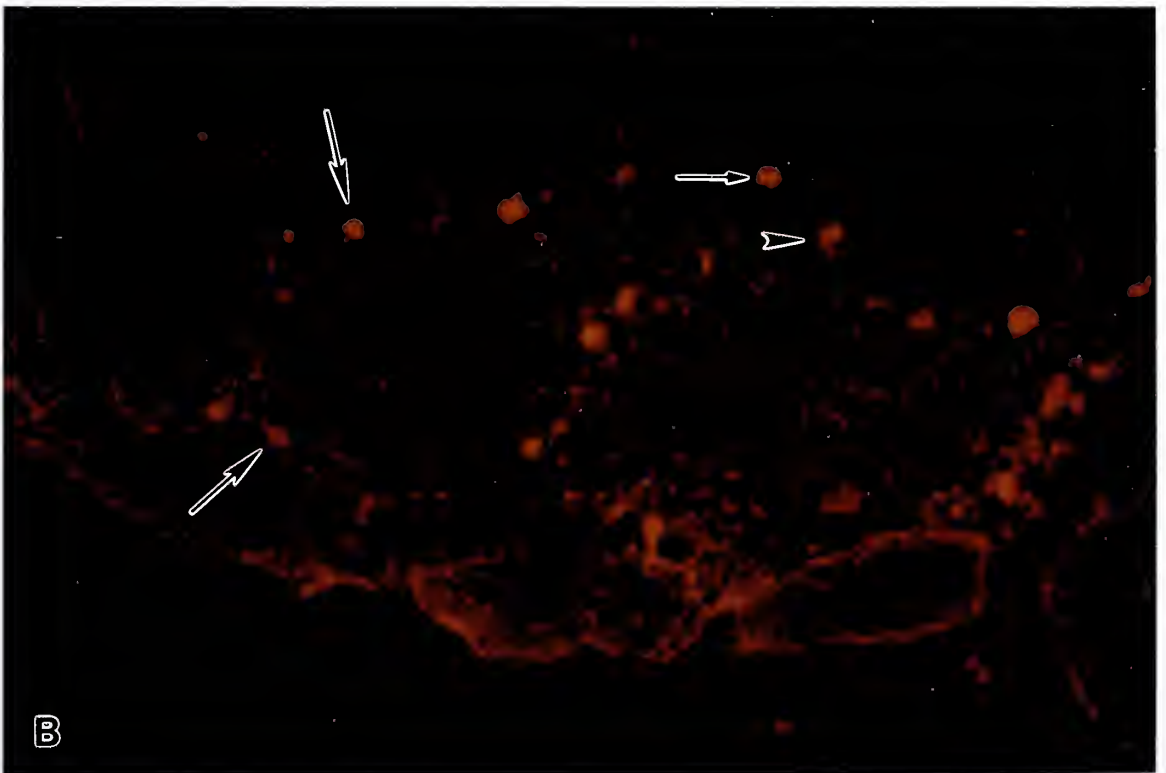
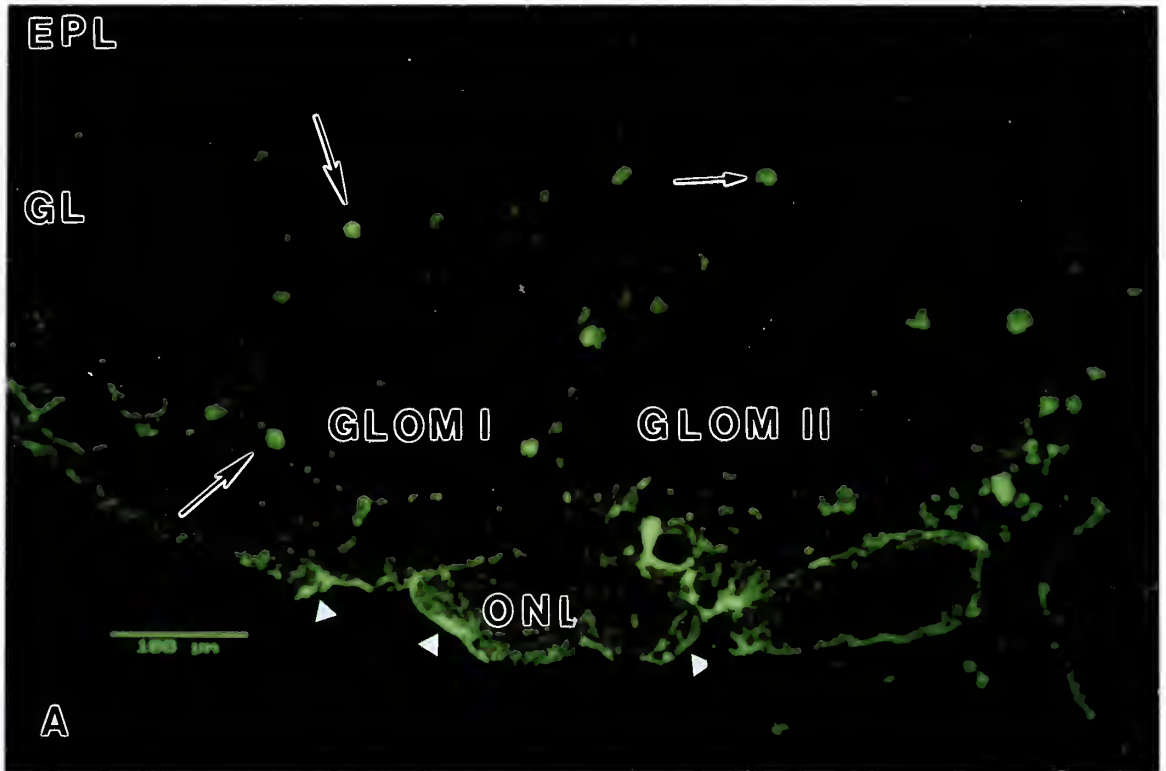








Figure 15. Higher magnification of rat olfactory bulb immunohistochemically stained using anti-GABA (A) and (B) anti-tyrosine hydroxylase primary antibodies. Notice individual glomeruli (GLOM) surrounded by periglomerular cells (arrows) and several superficial tufted cells (arrow head). Also present is a presumed superficial tufted cell within the external plexiform layer (smaller arrow) which is immunoreactive to GABA and tyrosine hydroxylase. Notice the granular pattern within the glomerulus in (B). This pattern could correspond to dendritic processes from periglomerular cells immunoreactive to tyrosine hydroxylase. Also evident in this figure is the presence of edge artifact (white arrow heads) which should not be confused with the more specific immunocytochemical staining. Abbreviations: ONL, olfactory nerve layer; GL, glomerular layer; EPL, external plexiform layer; GLOM I, glomerulus 1; GLOM II, glomerulus 2.





At higher magnification (Figure 15) individual glomeruli (GLOM I and GLOM II) were easily recognized by their cylindrical shape and well demarcated borders. Smaller GABA immunoreactive cell bodies (between 5-8 $\mu$ m) (arrows in Figure 15A) were confined to the periglomerular region most likely periglomerular cells. This is in contrast to larger cell bodies (between 10-12 $\mu$ m) (smaller arrow ) which were found predominantly in the glomerular-external plexiform layer border. The larger cell bodies were identified as potentially superficial short axon cells or superficial or middle tufted cells. The tyrosine hydroxylase immunoreaction produced an intraglomerular granular staining pattern suggestive of dopaminergic dendritic processes. Also present are individual periglomerular cells (arrows) and larger superficial tufted cells (arrow head). Notice that these superficial tufted cells were immunoreactive exclusively for tyrosine hydroxylase. It is appropriate to point out to the reader to avoid confusion the non specific edge artifact produced by the immunoreaction (white arrow heads) with the specific intraglomerular and cellular staining patterns. Figure 16B (stained with ethidium bromide) demonstrates the normal cellular density from individual glomeruli (GLOM I and GLOM II in Figure 16B) at the equivalent focal plane as for Figure 15. This cellular density included both glial and neuronal cells.

As has become evident from these figures, most cells seem to colocalize both GABA and tyrosine hydroxylase (see below under Colocalization). There were several instances where individual periglomerular cell were reactive for tyrosine hydroxylase exclusively (Figure 17 B, arrow head). These cells were identified as periglomerular cells based on their smaller cell body size as well as their location near the olfactory nerve layer (ONL in Figure 17A). Several dendritic processes arising from several tyrosine hydroxylase immunoreactive periglomerular cells were seen arborizing within glomeruli (small arrow). However, these dendrites could not be followed for a significant extent due to the antibody penetration or intermingling with several immunofluorescent fibers.





Figure 16. (A) Superimposed image of figures 15A and (B) demonstrating colocalization of GABA and tyrosine hydroxylase by yellow colored cell bodies (arrows). Arrow head, superficial tufted cell immunoreactive for tyrosine hydroxylase only; Smaller arrow, colocalizing tufted cell in the external plexiform layer. (B) Ethidium bromide staining representing same focal plane and field as (A) showing that only a portion of periglomerular cells are immunoreactive for either GABA or tyrosine hydroxylase. Notice that neuronal and glial elements are stained. Abbreviations: ONL, olfactory nerve layer; GL, glomerular layer; EPL, external plexiform layer; GLOM I, glomerulus 1; GLOM II, glomerulus 2.



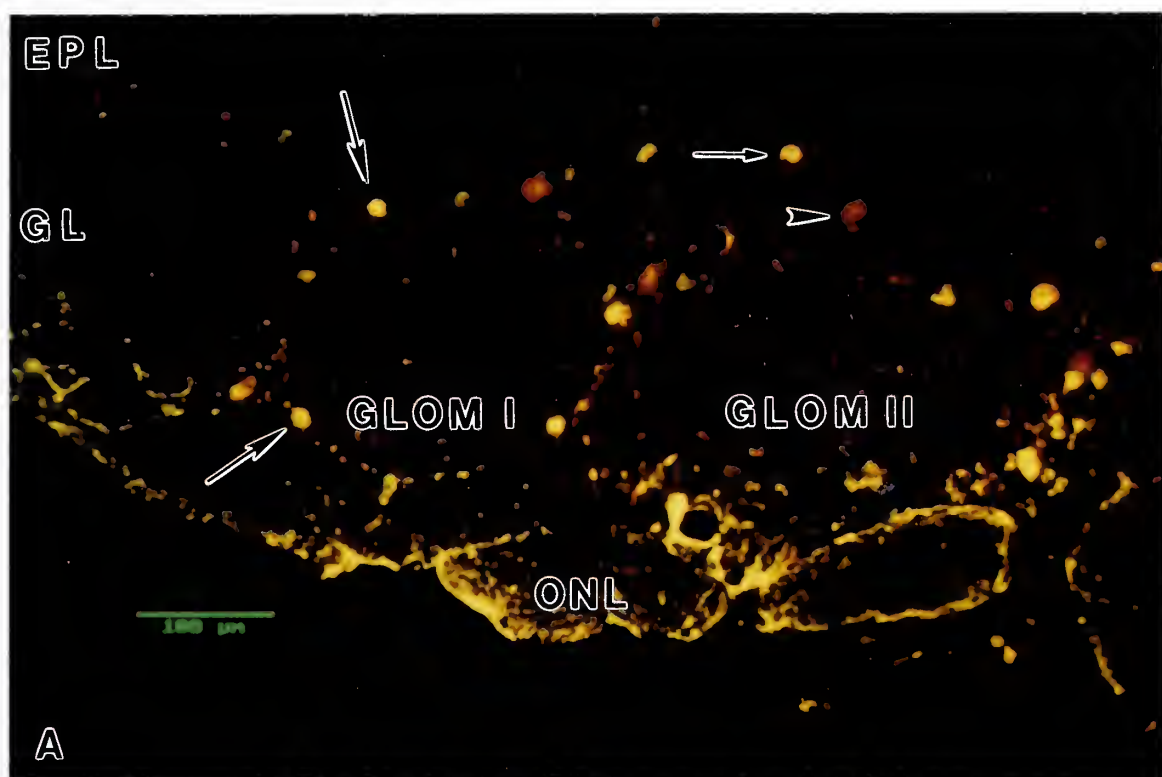






Figure 17. High magnification of rat olfactory bulb immunohistochemically stained using anti-GABA (A) and anti-tyrosine hydroxylase (B) primary antibodies. Periglomerular cell bodies (arrows) are found surrounding a glomerulus. Several periglomerular cells are immunoreactive to tyrosine hydroxylase only (white arrow head). Notice apical dendrites (smaller arrow) processes arising from periglomerular cells and directed toward the glomerulus. Of interest is the granular staining pattern in the external plexiform layer in A compared to the granular pattern in the glomerulus in B. The former likely represents granule cell dendritic processes terminating in the external plexiform layer while the latter corresponds to periglomerular and superficial tufted cells. Abbreviations: ONL, olfactory nerve layer; GL, glomerular layer; EPL, external plexiform layer; GLOM, glomerulus; white circle, blood vessel.

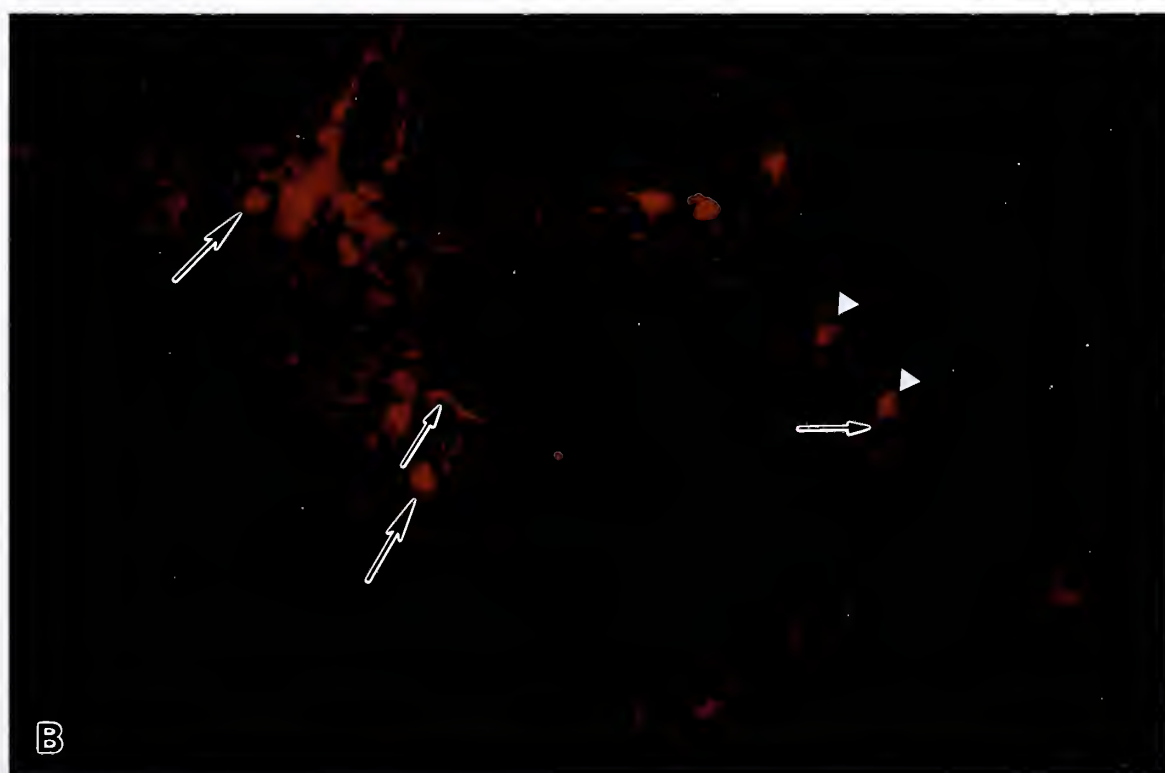
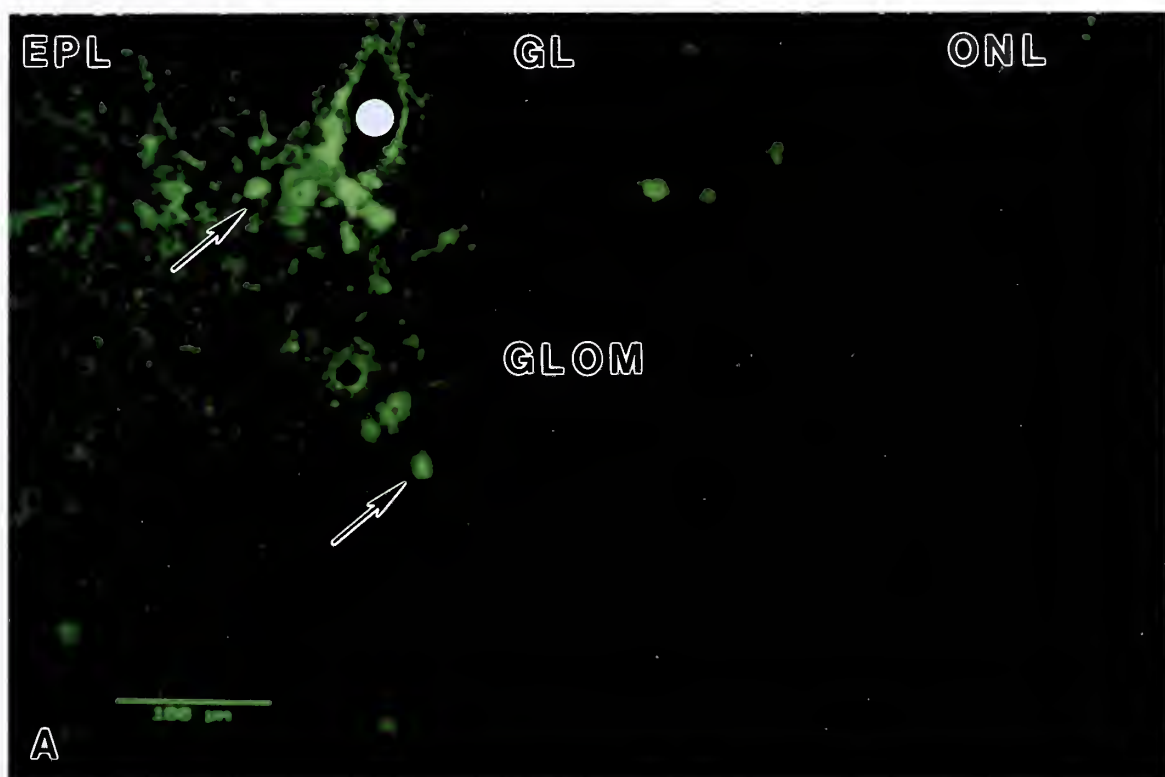






Figure 18. Superimposed image of figures 17 A and B demonstrating colocalization of GABA and tyrosine hydroxylase by yellow colored cell bodies (arrows). White arrow head, periglomerular cell immunoreactive to tyrosine hydroxylase only; White circle, blood vessel. Abbreviations: ONL, olfactory nerve layer; GL, glomerular layer; EPL, external plexiform layer; GLOM, glomerulus.



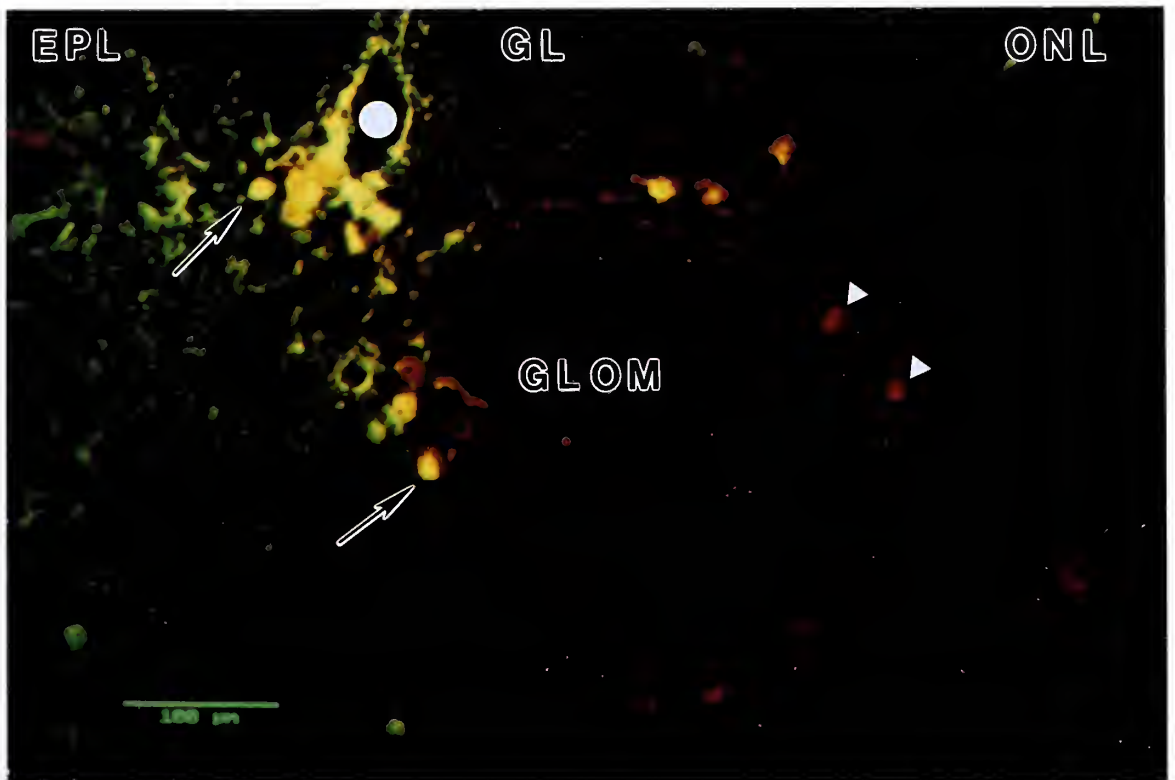






Figure 19. High magnification of rat olfactory bulb immunohistochemically stained using anti-GABA (A) and anti-tyrosine hydroxylase (B) primary antibodies. Periglomerular cell bodies (arrows) are found surrounding individual glomeruli. In A, a periglomerular cell is immunoreactive to GABA only (white arrow head). Also several immunoreactive cell bodies most likely superficial tufted cells are found in the external plexiform layer (double arrow heads). In B a superficial tufted cell immunoreactive to tyrosine hydroxylase is present in the glomerular/external plexiform layer border (arrow head). Abbreviations: ONL, olfactory nerve layer; GL, glomerular layer; EPL, external plexiform layer; GLOM I, glomerulus 1; GLOM II, glomerulus 2.

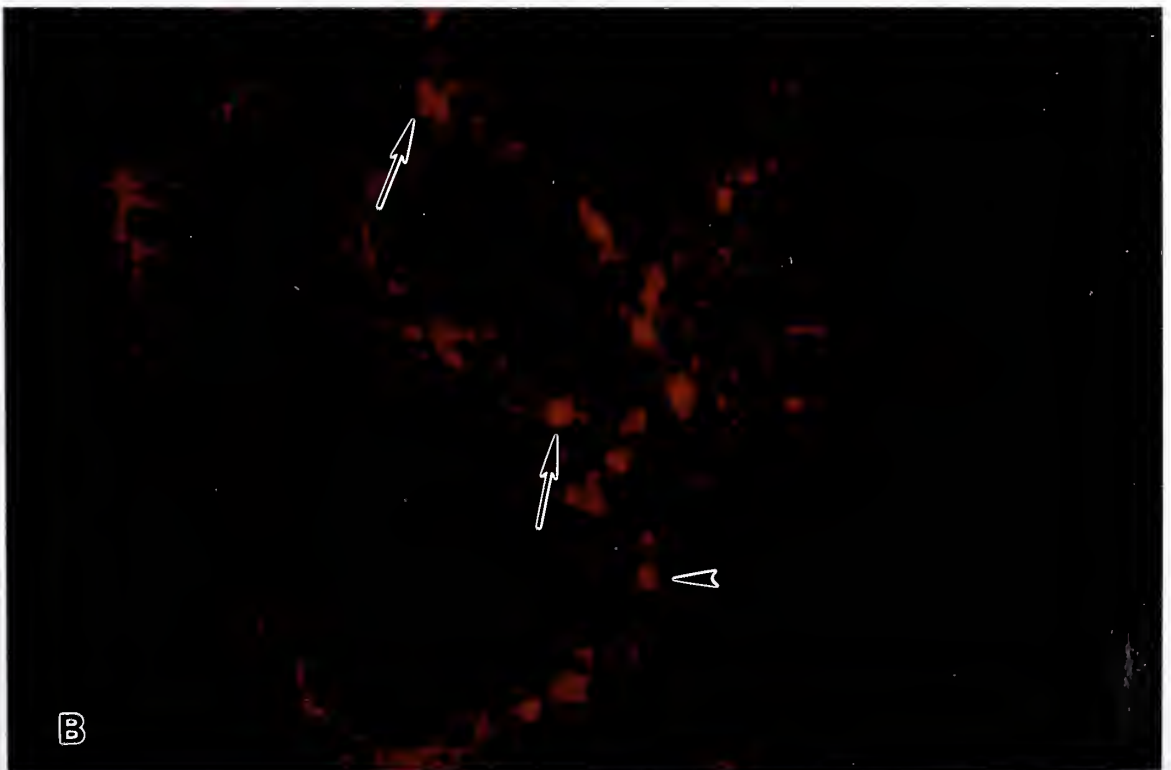
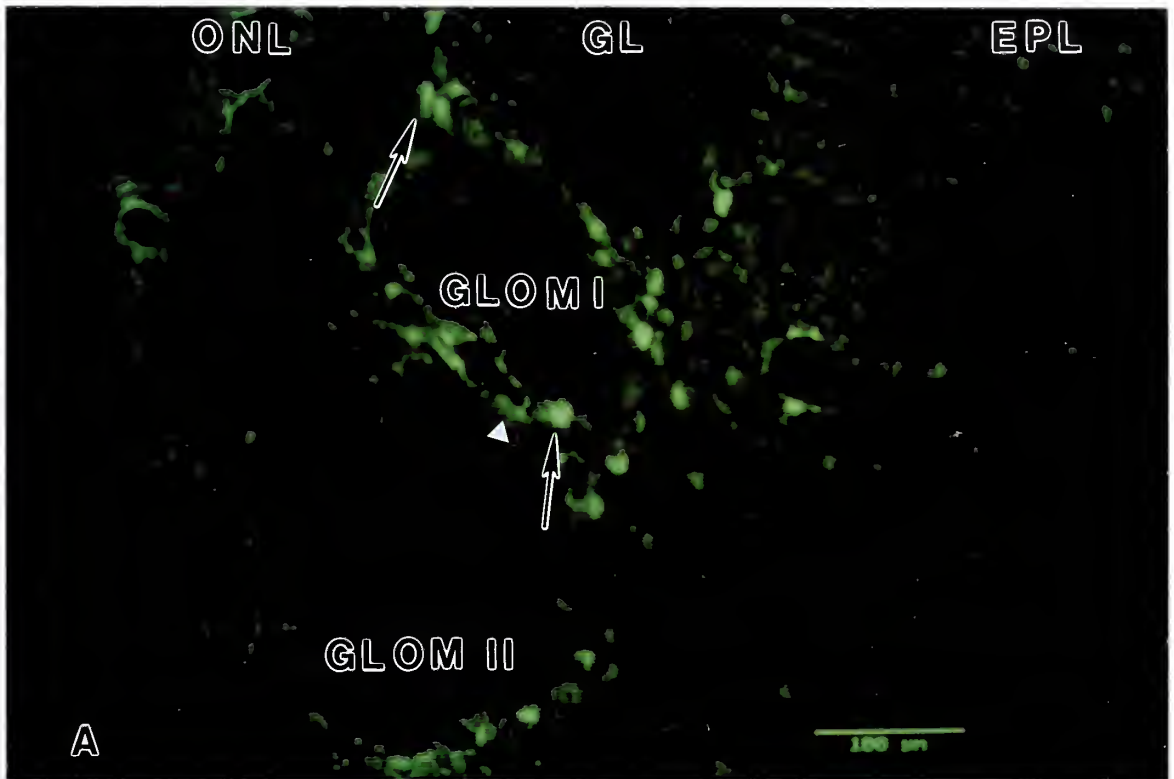


Figure 20. (A) Superimposed image of figures 19A and B demonstrating colocalization of GABA and tyrosine hydroxylase by yellow colored cell bodies (arrows). An individual periglomerular cell immunoreactive for GABA is localized within the periglomerular region (white arrow head). A superficial tufted cell immunoreactive for tyrosine hydroxylase is demonstrated by an arrow head while another superficial tufted cell colocalizing both GABA and tyrosine hydroxylase is demonstrated by double arrows. In B, ethidium bromide staining of cellular elements in the same field. Abbreviations: ONL, olfactory nerve layer; GL, glomerular layer; EPL, external plexiform layer; GLOM I, glomerulus 1; GLOM II, glomerulus 2.

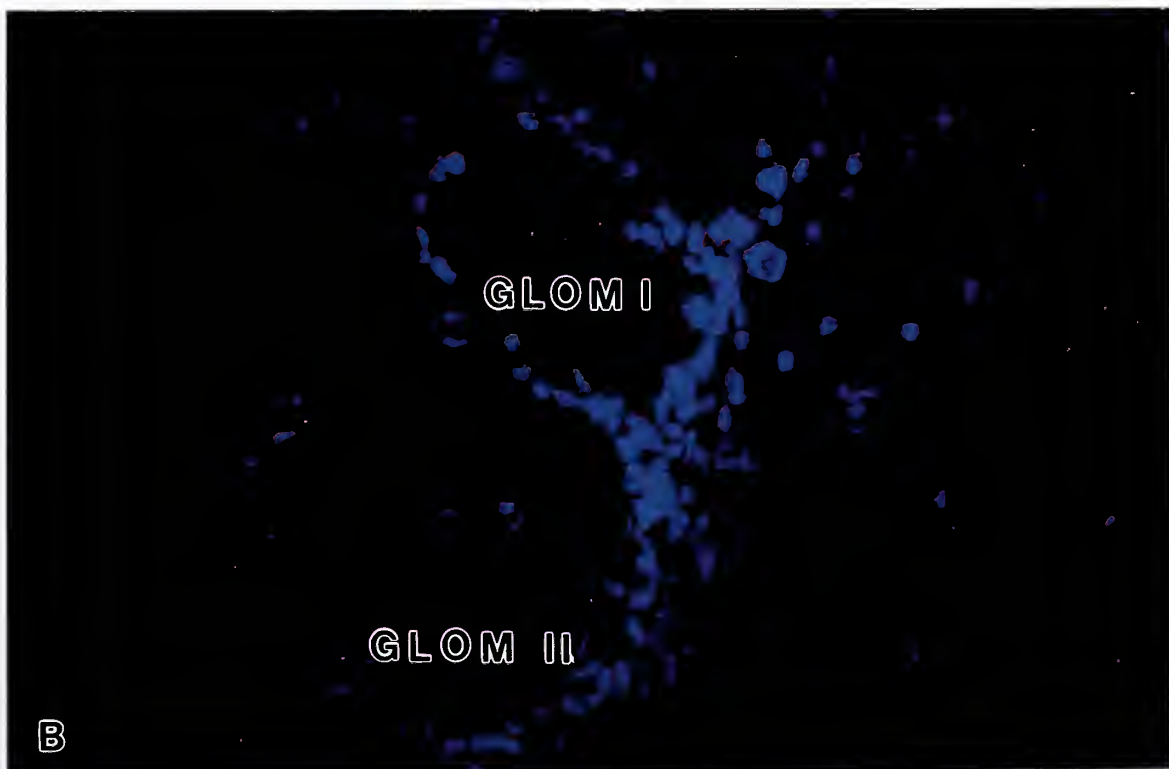
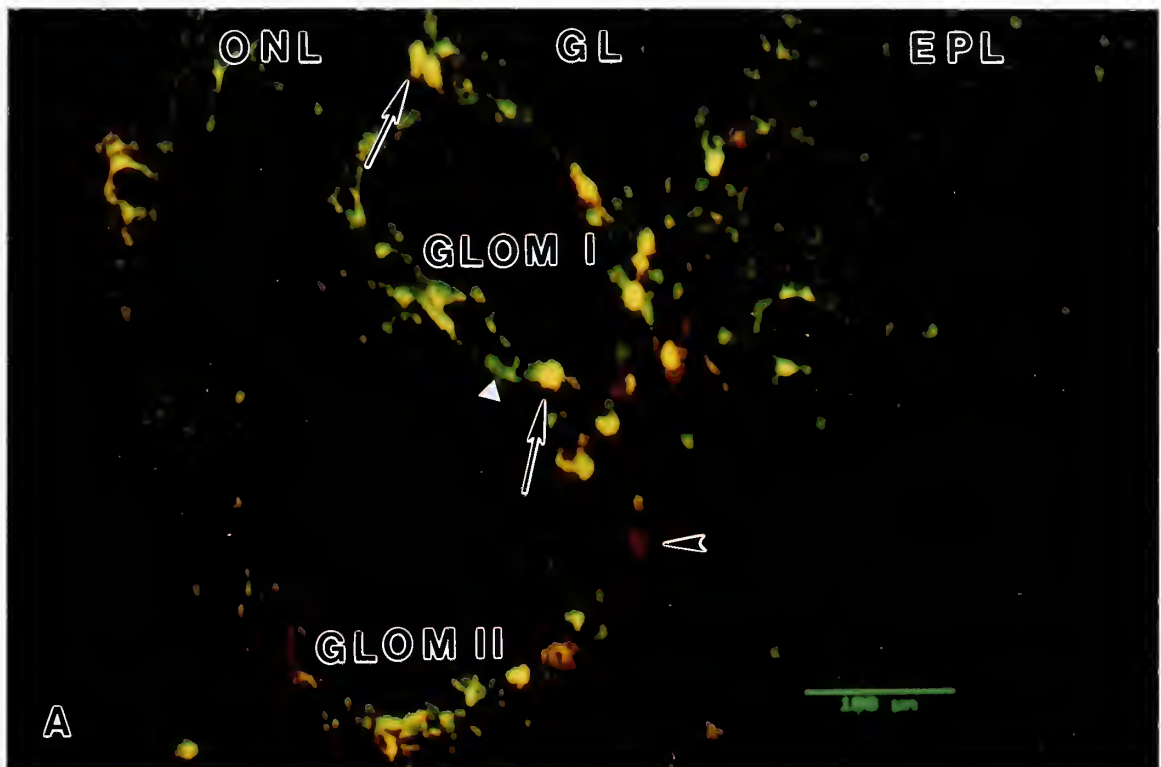








Figure 21. High magnification of rat olfactory bulb immunohistochemically stained using anti-GABA (A) and anti-tyrosine hydroxylase (B) primary antibodies. In A, two periglomerular cell bodies are immunoreactive for GABA only (white arrow heads). In both A and B, the periglomerular cells are distributed in the superficial aspect of a glomerulus near the olfactory nerve layer. This pattern is usually seen on glomeruli located in the medial portions of the olfactory bulb where the olfactory nerve and glomerular layer are the thickest. Arrow, individual periglomerular cell bodies. Abbreviations: ONL, olfactory nerve layer; GL, glomerular layer; EPL, external plexiform layer; GLOM, glomerulus.

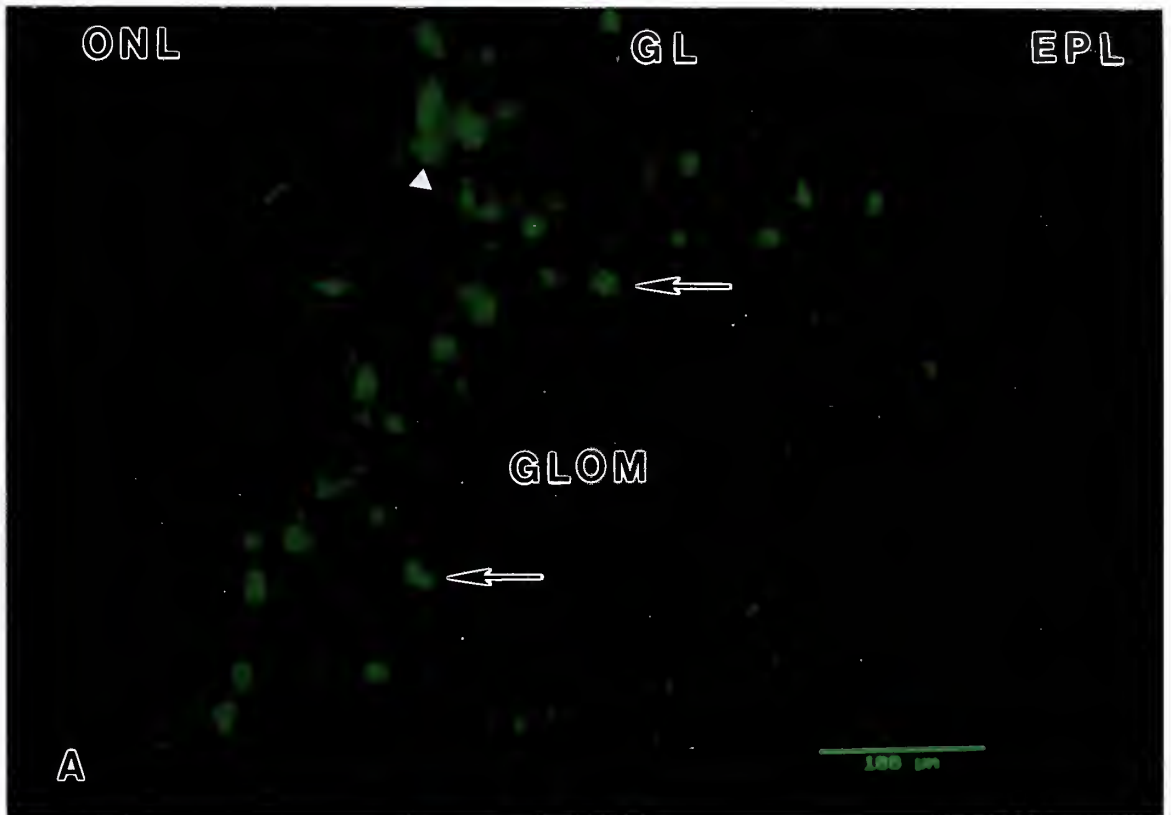
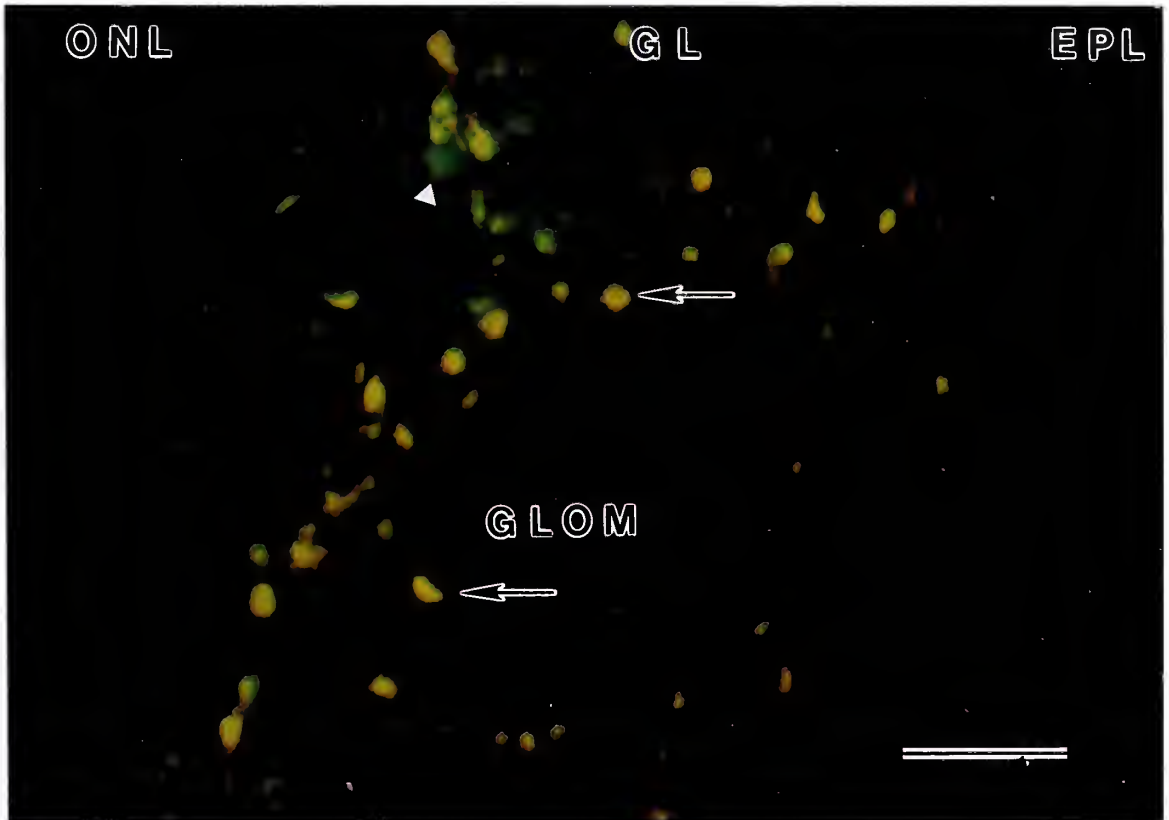






Figure 22. Superimposed image of figures 20A and B demonstrating colocalization of GABA and tyrosine hydroxylase by yellow colored cell bodies (arrows). White arrow heads demonstrate periglomerular cells immunoreactive for GABA only. Abbreviations: ONL, olfactory nerve layer; GL, glomerular layer; EPL, external plexiform layer; GLOM, glomerulus. Scale bar= 100 $\mu$ m.







Several periglomerular cell were exclusively immunoreactive for GABA as shown in Figures 19A and 21A (open arrow heads). Staining with ethidium bromide provided (Figure 20B) the normal cellular density for the identified glomeruli (GLOM I and GLOM II in Figure 20B).

### 3. Colocalization

One of the advantages of implementing confocal microscopy is the ability to superimpose two individual olfactory bulb fields. For example, we were able to acquire an image representative of tyrosine hydroxylase immunoreactive cell bodies and superimpose this onto the same GABA immunoreactive cellular field. The superimposed picture gave the GABA immunoreactive cells a green color, the tyrosine hydroxylase immunoreactive cells were red and cell that colocalized both neurotransmitters were seen as yellow. One of the difficulties in obtaining these images lies in controlling the gain and focal plane of section so as to minimize false positive immunoreactive cells.

At low magnification Figure 12 represents the superimposed images of Figure 11A and B. Notice colocalizing cell bodies with their yellow appearance (arrows) surrounding individual glomeruli. In the granule cell layer the majority of granule cell bodies stained for GABA and are evident by their green appearance. Figure 14 represents the superimposed images of Figure 13A and B. Notice periglomerular cells colocalizing both GABA and tyrosine hydroxylase, but in addition there are some cells (arrow head) that are exclusively immunoreactive for tyrosine hydroxylase or GABA (white arrow head) . Based on their location and larger cell bodies, the tyrosine hydroxylase (arrow head) were identified as superficial tufted cells.

At higher magnification (Figure 16A) periglomerular cells (arrows) colocalized tyrosine hydroxylase and GABA while superficial tufted cells (arrow heads) were immunoreactive for tyrosine hydroxylase. In the external plexiform layer (EPL) a larger cell also colocalizes tyrosine hydroxylase and GABA (small arrow). Based on its



location and morphology this cell could be classified as a superficial short axon or superficial tufted cell. A cluster of colocalizing periglomerular cells adjacent to a blood vessels (asterisk) are shown in Figure 18. Notice individual periglomerular cells immunoreactive for tyrosine hydroxylase (arrow head) near the olfactory nerve layer . Of interest is the granular immunoreactive pattern for tyrosine hydroxylase usually found within the glomerulus while a similar pattern for GABA is found in the external plexiform layer . This granular pattern might be indicative of granule cell dendritic processes terminating throughout the external plexiform layer. Some periglomerular cells were found to immunoreact exclusively for GABA as shown in Figure 20 A and 22 (white arrow head). Also present in Figure 20 A is a superficial tufted cell immunoreactive to tyrosine hydroxylase (arrow head). Figure 20 B suggests that only a portion of periglomerular cells are immunoreactive for either GABA or tyrosine hydroxylase.

#### 4. Quantitative Analysis

The cell counts represented pooled total counts in limited tissue fields encompassing three and a half olfactory bulb sections. The majority of the immunoreactive neurons within the glomerular layer colocalized both GABA and tyrosine hydroxylase. Of the 4,354 immunoreactive neurons 3,126 (71.8%) contained both tyrosine hydroxylase and GABA, 1,158 (26.6%) GABA only, and 70 (1.6%) tyrosine hydroxylase only. The majority of the colocalizing neurons had a small size and dendritic processes stained extending into individual glomeruli. These cells were identified as periglomerular cells based on their location and morphological characteristics. The majority of neurons immunoreactive for GABA were also identified as periglomerular cells based on their location and morphology. On the other hand, the tyrosine hydroxylase immunoreactive cells varied in their morphological size and location. For the most part larger cells localized to the glomerular-external plexiform



layer border. These cells corresponded to superficial tufted cells. Of the 105 superficial tufted cells identified, 19 (18.1%) were immunoreactive to tyrosine hydroxylase only, 86 (81.9%) colocalized both GABA and tyrosine hydroxylase, and none were immunoreactive for GABA only.

In an attempt to shed some insight into the percentage of periglomerular cells immunoreactive for GABA and/or tyrosine hydroxylase in a given olfactory bulb section, several sections previously stained with cresyl violet were used. These sections were 10 $\mu$ m thick, thus approximating the depth of antibody penetration. Also, these olfactory bulb sections corresponded to the areas where the immunocytochemistry studies were performed using the same magnification. The cell counts were made in the periglomerular region. From the cresyl violet observations it was roughly approximated that in a 10 $\mu$ m thick olfactory bulb section there is an average of 6,151 cells surrounding the glomeruli. These cells include glial, short axon, superficial tufted, and periglomerular cells. This corresponds to approximately 21,529 cells in three and a half olfactory bulb sections 10 $\mu$ m thick. Combining the immunoreaction and cresyl violet stained cell counts as a very rough estimate, 0.33% (70/21,529) of periglomerular cells are tyrosine hydroxylase only, 5.0% (1,158/21,529) are GABA only, and 14.5% (3,126/21,529) colocalize both GABA and tyrosine hydroxylase. Again it should be emphasized that this method of cell counting is not the most appropriate one for this study (see below under Discussion section) and the total count in the cresyl violet section include several populations of cells other than periglomerular cells.

## F. SUMMARY

The purpose of this study was to examine the differential distribution of olfactory bulb neurons, in particular periglomerular cells, immunoreactive for tyrosine hydroxylase and GABA. Tyrosine hydroxylase immunoreactive cell bodies have a differential distribution in the olfactory bulb. These immunoreactive cell bodies are found mostly in





the glomerular layer, with few scattered ones in the superficial aspect of the external plexiform layer. The small size and dendritic extensions into individual glomeruli of tyrosine hydroxylase immunoreactive cell bodies correspond to periglomerular cells. Larger cell bodies located predominantly in the superficial aspect of the external plexiform layer were identified as superficial tufted cells. Among the periglomerular cells, there was no apparent differential distribution in individual glomeruli. That is, immunoreactive periglomerular cells were found between glomeruli, near the surface where the olfactory nerve enters the glomerulus, and in the glomerular-external plexiform layer border. However, immunoreactive tufted cell bodies were limited to the deeper aspect of the glomerular-external plexiform layer border area.

GABA immunoreactive cell bodies were located predominantly in two regions of the olfactory bulb, the glomerular and granule cell layer. Compared to tyrosine hydroxylase there was a higher number of scattered immunoreactive cell bodies within the external plexiform layer, most likely corresponding to short axon cells. The cell bodies located in the glomerular layer were identified as periglomerular cells as well as several examples of superficial tufted cells that were found to be immunoreactive for GABA. In the granule cell layer the immunoreactive cell bodies corresponded to granule cells. Similar to tyrosine hydroxylase, GABA immunoreactive periglomerular cells did not exhibit a differential distribution within individual glomeruli.

The majority of immunoreactive periglomerular cell bodies were found to colocalize both GABA and tyrosine hydroxylase (71.8%). There was a higher percentage of periglomerular cells immunoreactive for GABA only (26.6%) compared to tyrosine hydroxylase only (1.6%). Superficial tufted cells were preferentially immunoreactive for tyrosine hydroxylase only (18.1%) compared to GABA only (0%), but the majority were colocalizing (81.9%) both GABA and tyrosine hydroxylase. These percentages differ drastically when comparing them with the normal density of periglomerular cells. Based on this study it is evident that there are at least three subpopulations of periglomerular





cells: 1) those immunoreactive to tyrosine hydroxylase exclusively; 2) those immunoreactive to GABA exclusively; and 3) those that colocalize both. In addition there may be further periglomerular cells not detected in this study that are not immunoreactive to either GABA or tyrosine hydroxylase. It is enticing to speculate that the immunocytochemically heterogeneous population of periglomerular cells entails an equivalent heterogeneous synaptic distribution and local circuit organization. This postulate serve as a foundation for Study III (see below).



### **III. STUDY II-ELECTRON MICROSCOPIC ANALYSIS OF GLOMERULAR PROFILES**

#### **A. PURPOSE OF STUDY**

Electron microscopy at the present time remains as the only technique capable of providing a detailed and definitive description of synaptic connections in any area of the brain. Extensive work employing serial reconstructions have characterized the structural and synaptic composition of the glomerular neuropil (Pinching and Powell, 1971a, b; White, 1971, 1972). Therefore, the purpose of this study is not to provide a detailed anatomical description but rather characterize and elucidate patterns or the topography of synaptic organization within the olfactory bulb glomerulus. This characterization served as a foundation for understanding principles of synaptic organization within individual glomeruli observed in the next study (see below).

#### **B. TISSUE COLLECTION**

Two adult Sprague Dawley male rats (180-300 grams) were euthanized and perfused as outlined in Study I. The perfusate concentration consisted of 4% paraformaldehyde and 2% glutaraldehyde. Following perfusion the brains were removed and processed as described in Study I.

Following post-immersion fixation the brains were coronally blocked to include all layers of the olfactory bulb along the rostro-caudal axis. The tissue received a 3 X 15 minute washes of 0.1 M phosphate buffer, was post fixed in 2% osmium in 0.1M phosphate buffer for 1 hour, dehydrated in 50% and 70% ETOH, stained with uranyl acetate in 70% ETOH for 1 hour, dehydrated through grade alcohols to absolute alcohol and embedded for coronal sectioning in Epon. The tissue was blocked to obtain coronal sections of the medial portion of the olfactory bulbs where the glomeruli are consistently large and spherical. Thick 1 $\mu$ m sections that included the olfactory nerve layer and



external plexiform layer were obtained and stained with toluidene blue. These sections were utilized for orientation prior to thin sectioning. After each thick section, thin silver, sections, approximately 70nm were obtained on a Reichert Ultracut E Microtome and mounted on Formvar coated, 1 X 2mm slot grids. After collecting 6 grids the tissue was trimmed between 200-250 $\mu$ m along the rostro-caudal axis. Another 1 $\mu$ m thick section was obtained and new glomeruli selected for analysis. Once a new set of glomeruli was confirmed another 4 grids containing thin section were collected. This process was repeated 7 times and approximately 10-15 glomeruli were collected. The grids were lightly stained with lead citrate (Reynolds, 1961) and examined in a JEOL 1200 Electron Microscope.

### C. DATA AQUISITION AND ANALYSIS

Individual glomeruli from the thick sections were selected based on their large and spherical appearance. Fudicial points, such as blood vessels surrounding chosen, glomeruli were identified in thick sections with the light microscope, and courses or montages were outlined that spanned the entire glomerulus. At the level of the electron microscope the fudicial points were identified and transglomerular montages were obtained. The montages extended from one fudicial point to another. In addition to these fudicial points, periglomerular cell bodies surrounding the glomerulus aided in establishing the beginning and terminations of montages. Figure 23 illustrates the manner in which the transglomerular montages were obtained. Photomicrographs were taken at a primary magnification of X12,000 and exposure time of 2 mamp/sec. The photomicrographs were printed on Kodak P2 paper for a final printed magnification of X30,000. Transglomerular montages were mounted together and consisted of approximately 25-45 photomicrographs. A qualitative assessment from each probe was obtained paying particular attention to the ultrastructural and synaptic organization of olfactory nerve terminals and the mitral/tufted and periglomerular cells dendritic profiles .



The kind help of Kerry Arsenault was greatly appreciated in obtaining, developing, printing, and mounting all the glomerular montages described above.

## D. RESULTS

Figure 24 illustrates approximate areas within the glomerulus Figures 25-36 represent.

### 1. The Glomerulus

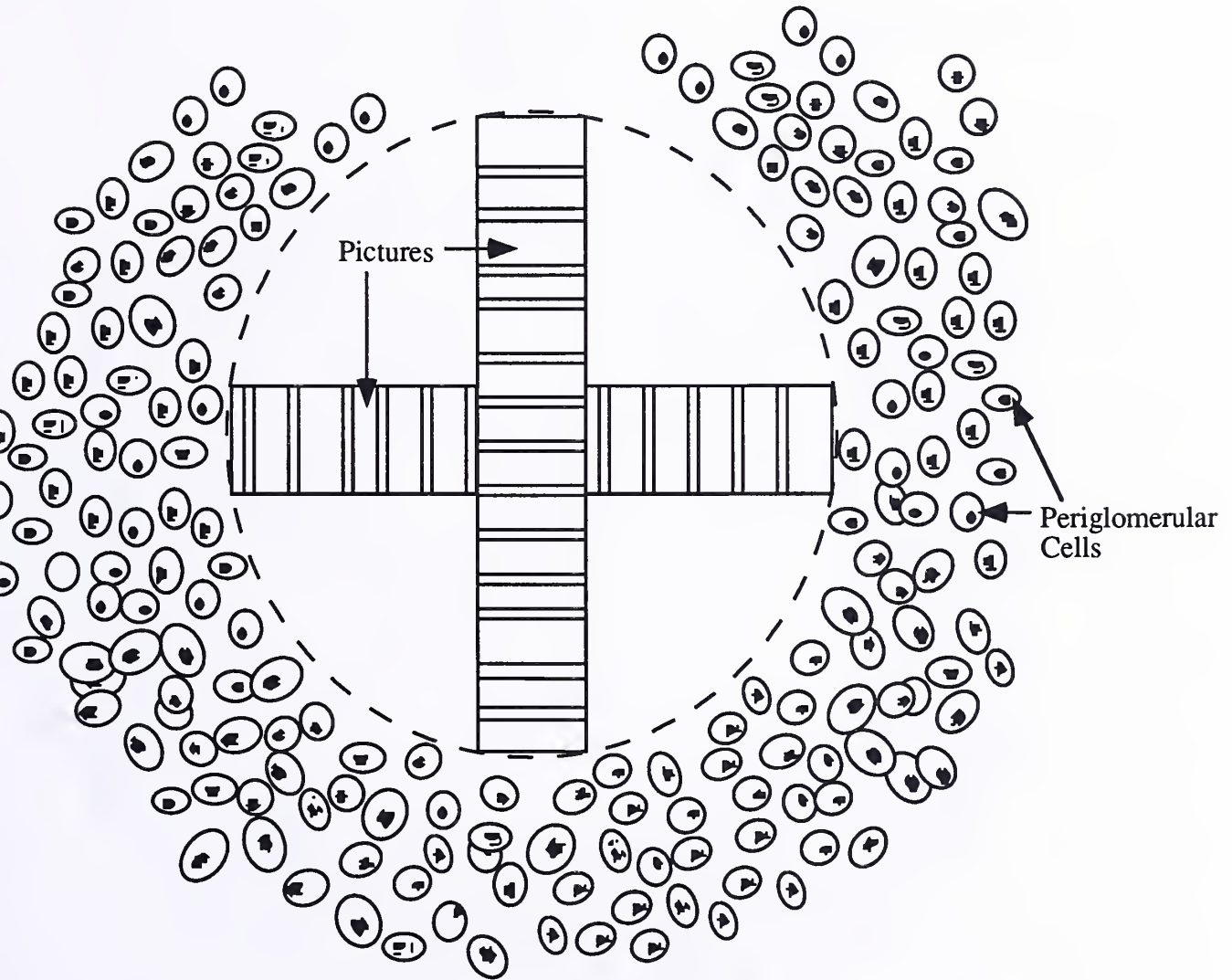
The size and shape of the glomeruli were very variable. Generally they are spherical, oval or pear-shaped with a mean internal diameter ranging from 30 to 200 $\mu$ m. In the olfactory nerve-glomerular layer border the olfactory nerve axons are traveling in a rostro-caudal direction in very tightly packed fascicles . As seen in figure 25 these axons are characteristically unmyelinated with an approximate cross sectional diameter of 0.2 $\mu$ m. Individual fascicles enter each glomeruli from a variety of directions and sites. Although most enter from the superficial aspect of the glomeruli evident by longitudinal cut axons (arrows) as seen in Figure 26.

Surrounding individual glomeruli are the cell bodies of glial cells, periglomerular cells, short axon cells and in the deeper portions superficial tufted cells. The vast majority of cell bodies are those belonging to periglomerular cells (PG in Figure 27). Periglomerular cells are characterized by their small size (5-8 $\mu$ m), thin rim of cytoplasm surrounding the nucleus (between arrow heads), and intranuclear invaginations (arrows). Deeper, in the external plexiform-glomerular layer border, superficial tufted cells (TC in Figure 28) are found with larger cell bodies (10-15 $\mu$ m) and a thicker rim of cytoplasm surrounding the nucleus (in between arrow heads) with numerous cytoplasmic organelles. Glial cell bodies (GL in Figure 29) were smaller in size (4-6 $\mu$ m) and had a characteristically darker nucleus and cytoplasm filled with glycogen granules. The glial cells were distributed throughout the periglomerular region and seldomly within a





## OLFACTORY NERVE LAYER

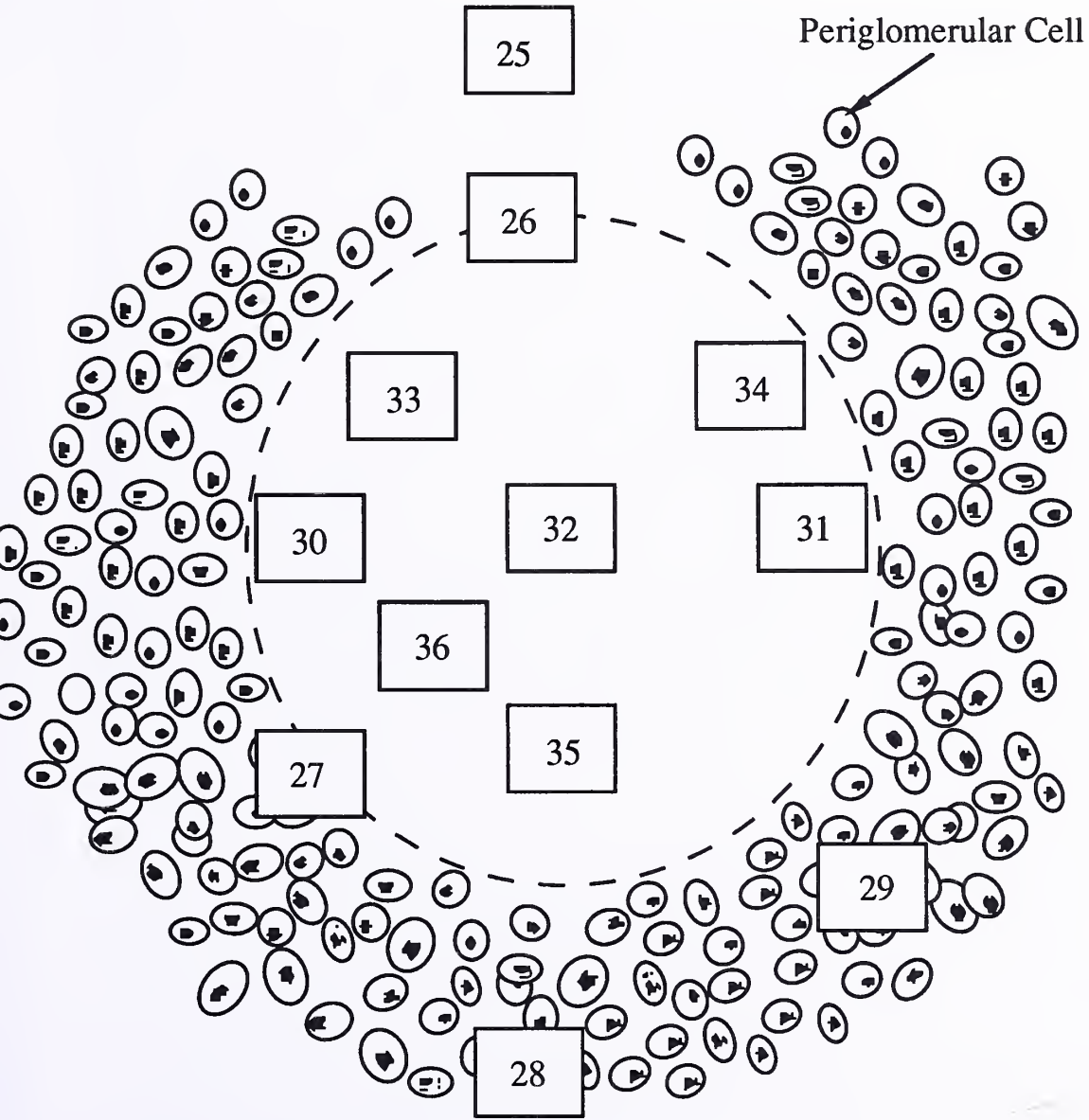


## EXTERNAL PLEXIFORM LAYER

Figure 23. Illustration of how transglomerular montages were obtained. Individual glomeruli are surrounded by many periglomerular cell bodies. Photomicrographs extended from one side of the glomerulus to another in their longest cross sectional dimensions. Cell bodies outlined the probes margin.



## OLFACTORY NERVE LAYER



## EXTERNAL PLEXIFORM LAYER

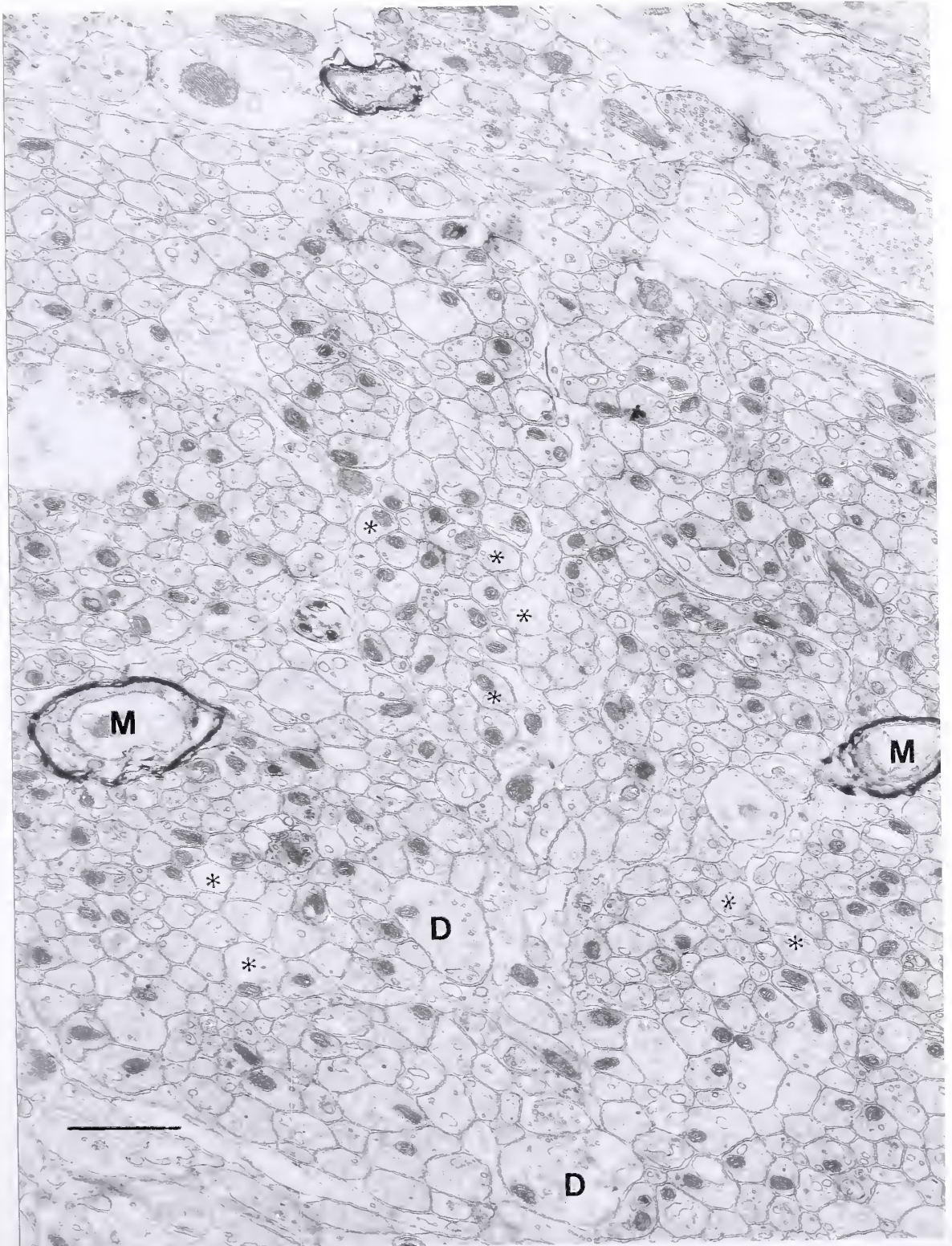
Figure 24. Illustration showing approximate location or areas for Figures 25-36 representing individual glomeruli.





Figure 25. Electron micrograph taken in the olfactory nerve layer. Notice the cross sectionally cut, small caliber ( $0.2\mu\text{m}$ ) unmyelinated olfactory receptor cell axons (asterisks) tightly packed. Several large dendritic processes (D) and myelinated axons (M) are found among these axons. Scale bar=  $1\mu\text{m}$ .











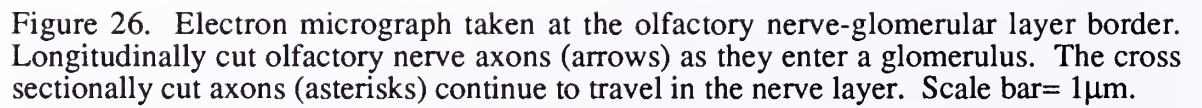
The image is an electron micrograph showing the interface between the olfactory nerve and the glomerular layer. It displays several axons in different orientations. Some axons are cut longitudinally, appearing as thin, parallel lines, and are indicated by arrows. Other axons are cut cross-sectionally, appearing as small, dark, circular or oval profiles, and are indicated by asterisks. The background shows the fine structure of the nerve and glomerular layers.

Figure 26. Electron micrograph taken at the olfactory nerve-glomerular layer border. Longitudinally cut olfactory nerve axons (arrows) as they enter a glomerulus. The cross sectionally cut axons (asterisks) continue to travel in the nerve layer. Scale bar= 1 $\mu$ m.

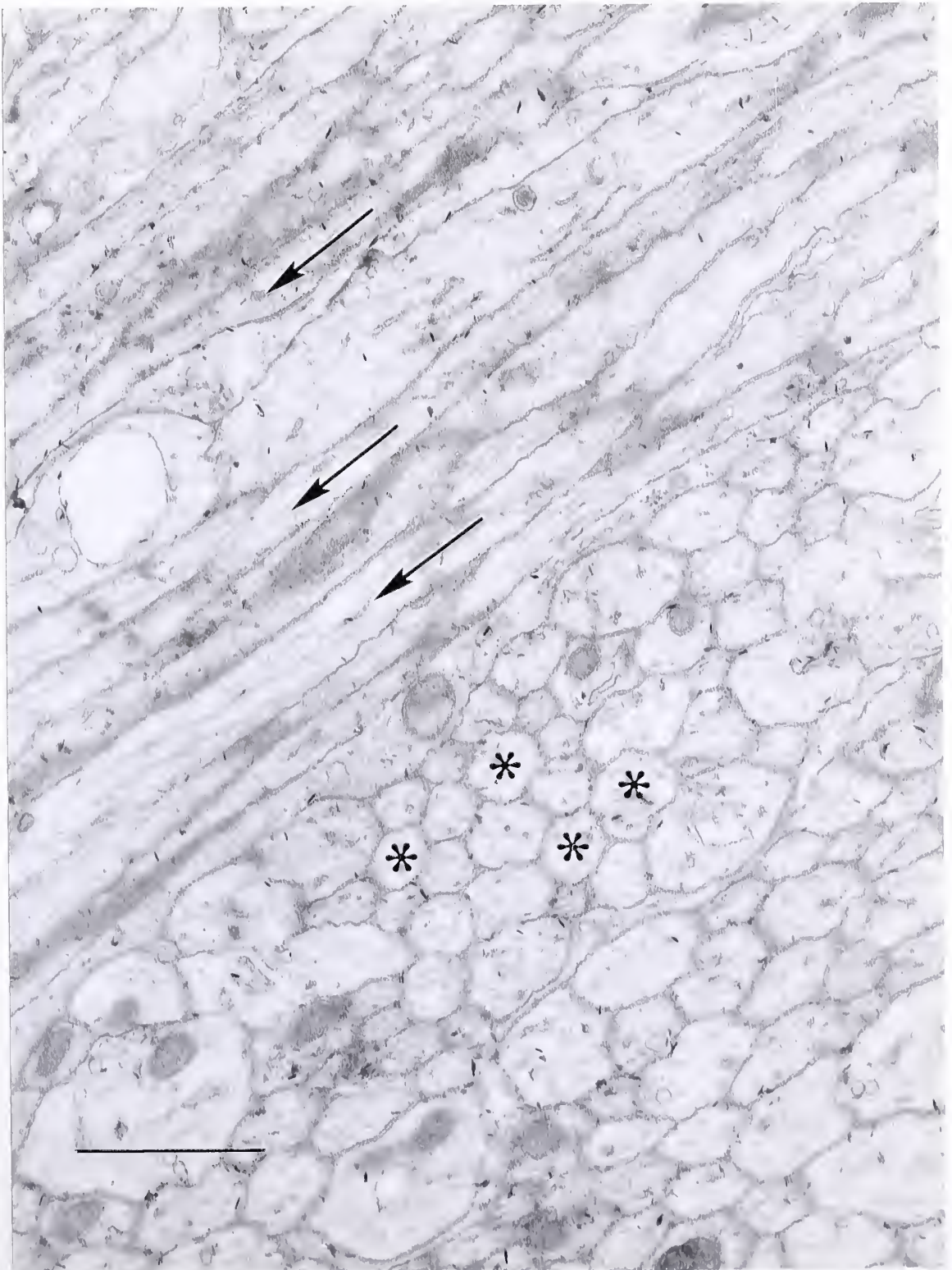






Figure 27. Electron micrograph of two periglomerular cell bodies. Notice thin rim of cytoplasm surrounding nucleus (between arrow heads) and intranuclear invaginations (arrows). These and the small size are typical characteristics of periglomerular cell bodies. Also notice large dendritic processes in the vicinity of these cells. Abbreviations: PG, periglomerular cells; D, dendrites; NUC, nucleus. Scale bar= 1 $\mu$ m.



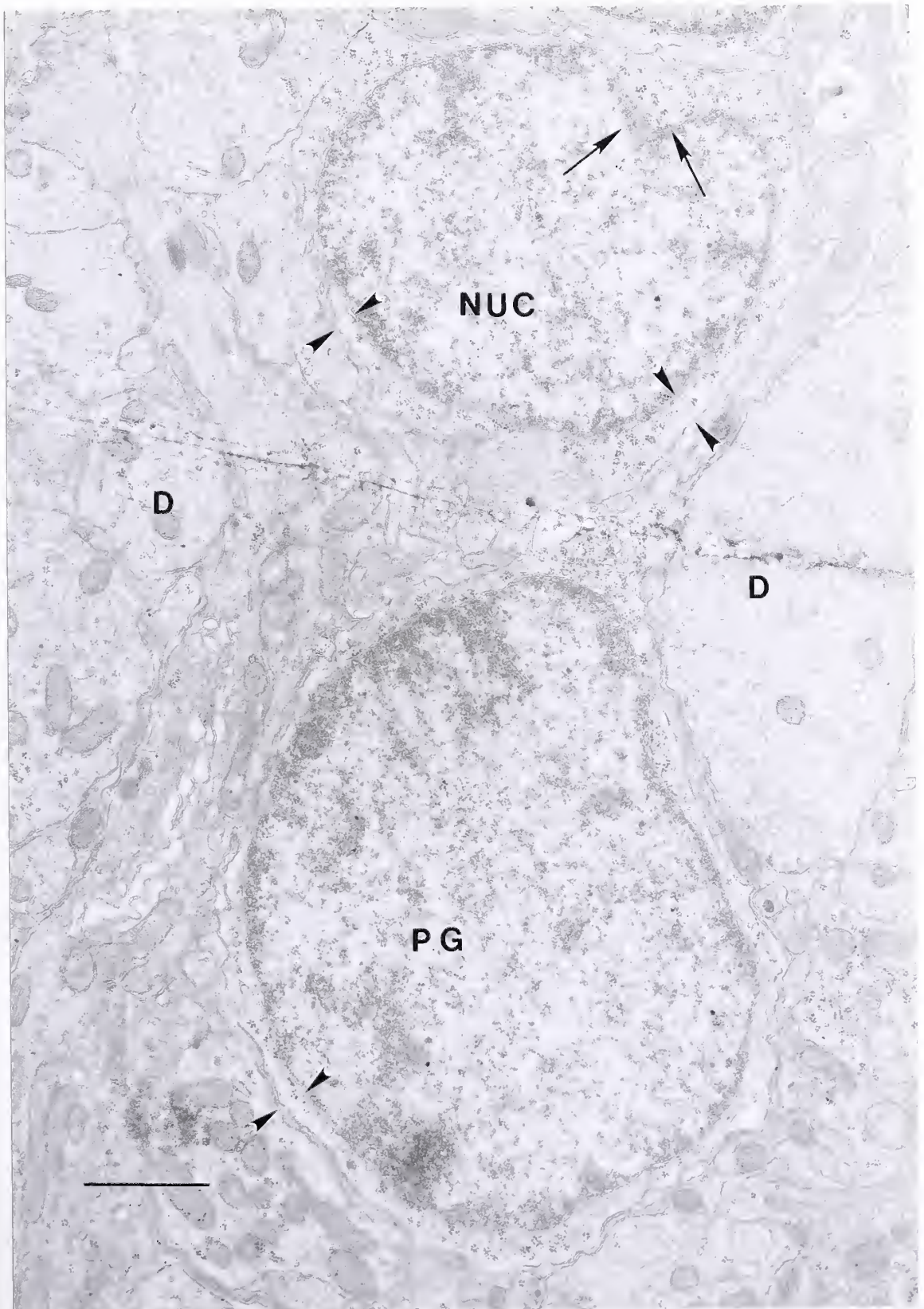








Figure 28. Electron micrograph taken in the external plexiform-glomerular layer border. The larger cell body belongs to a tufted cell while the smaller to a periglomerular cell. Notice the different thickness of cytoplasm surrounding the nucleus of each cell (between arrow heads). The tufted cell cytoplasm also contained numerous organelles such as mitochondrion, ribosomes and endoplasmic reticulum (open arrow heads). Tufted cells also contain intranuclear invaginations (arrows). Abbreviations: PG, periglomerular cells; TC, tufted cell. Scale bar= 2 $\mu$ m.

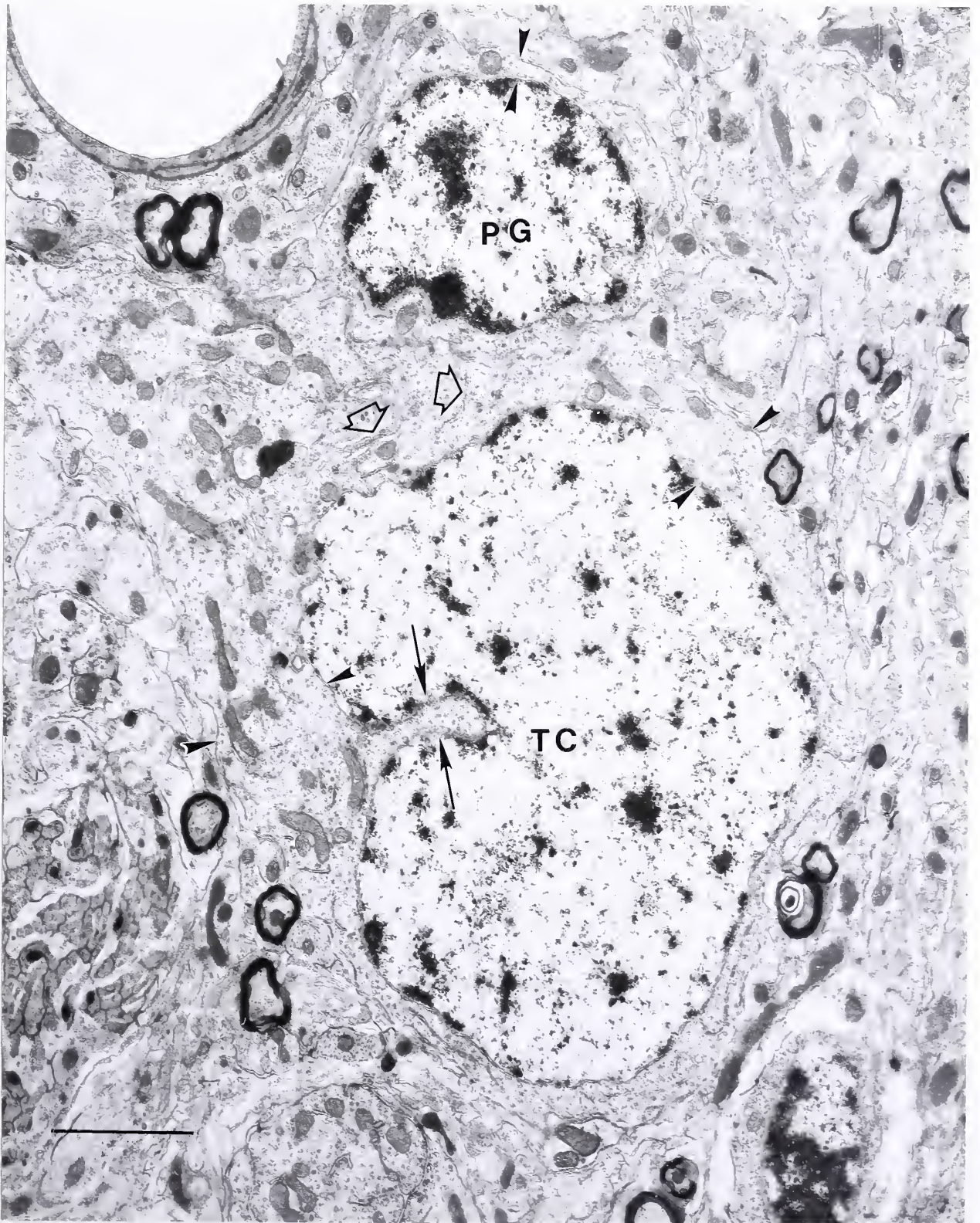
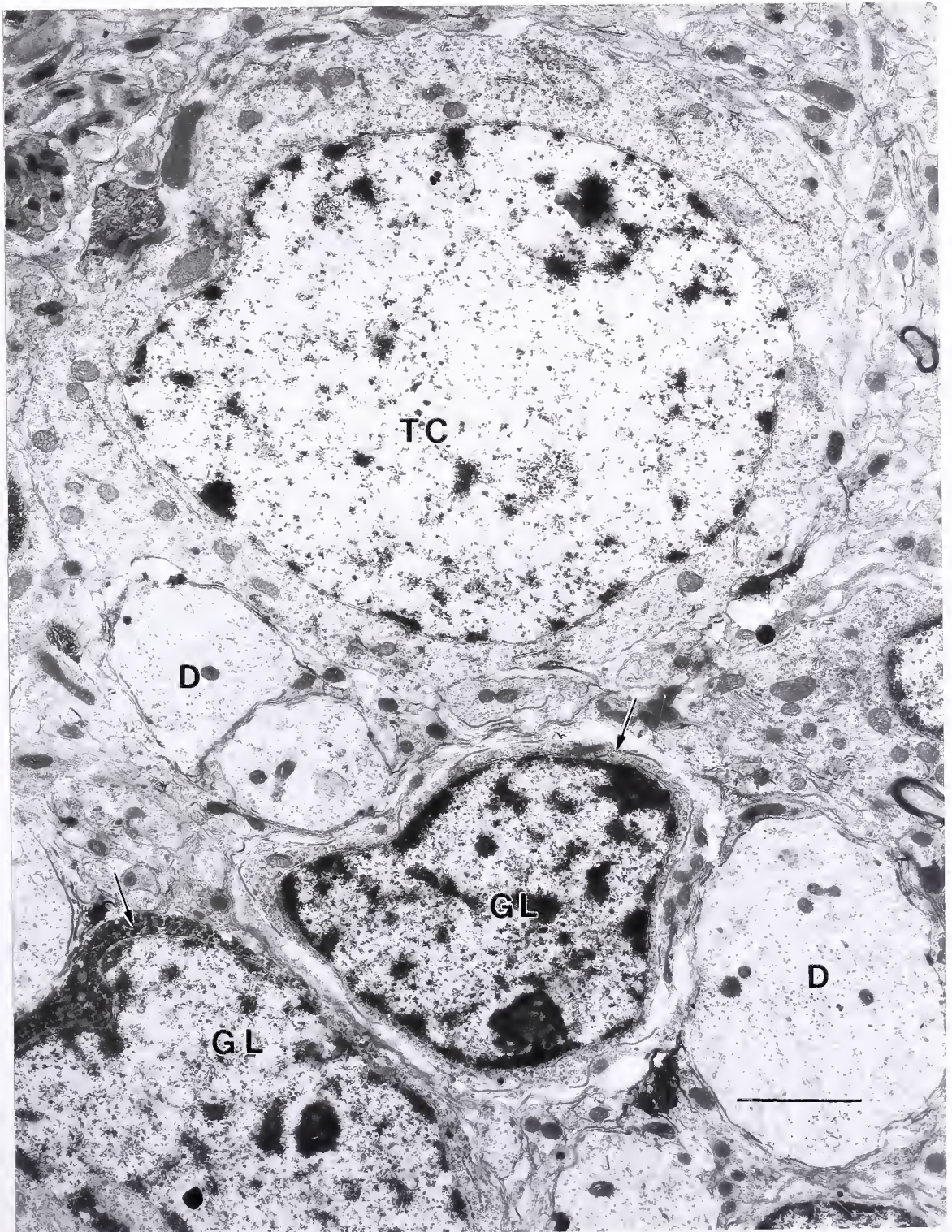






Figure 29. Electron micrograph taken in the external plexiform-glomerular layer border. The larger cell body belongs to a tufted cell while the smaller ones to glial cells. Glial cells are characterized by a darker cytoplasm (arrow) containing numerous glycogen granules, also the nuclei of these cells are heterogeneously darker compared to other cells. Abbreviations: GL, glial cells; TC, tufted cell; D, dendrites. Scale bar= 2 $\mu$ m.









glomerulus. Short axons cells (not shown) were intermediate between periglomerular and superficial tufted cells and seldomly visualized in the periglomerular region.

It is important to recognize that the olfactory bulb presents an ideal configuration for studying principles of synaptic organization since it is extremely simple to identify neuronal processes. The olfactory nerve terminals are very distinctive in their morphology (see below). The unique morphological characteristics facilitate discrimination between axon and dendrites and their synaptic organization. Despite this simplicity in identification, it is important to outline the morphological differences between axons and dendrites. Axon and dendrites can contain many vesicles within their axoplasm and be either pre-or postsynaptic. Dendrites for the most part have varying sizes and very irregular outlines compared to the more cylindrical and smaller axons. Frequent ribosomal rosettes and cisterna of endoplasmic reticulum in dendritic profiles are useful but not definitive criteria for distinguishing a dendrite. Also, dendrites usually contain vesicles over a large part of their profiles and often exhibit more electron dense microtubules compared to axons.

Interspersed within the glomerulus there are numerous islets composed of olfactory nerve terminals and pale dendritic profiles belonging to mitral/tufted and periglomerular cells (Figures 30 and 31). Dendritic islets varied in size, were composed of longitudinally and cross sectionally cut dendritic processes, and were surrounded by olfactory nerve terminals. The size of dendritic islets seems to be dependent on the size of individual dendritic processes forming them. That is, larger islets have larger dendrites and vice versa. Grossly, the synaptic density seemed to correlate with the size of dendritic profiles. There was a tendency to see more axodendritic and dendrodendritic synapses in those islets where the individual dendrites were smaller in dimension.

By examining individual montages one can roughly divide them into outer and inner glomerular divisions where the outer is the shell and the inner is the core of the glomerulus. Although a quantitative synaptological analysis is underway, from a






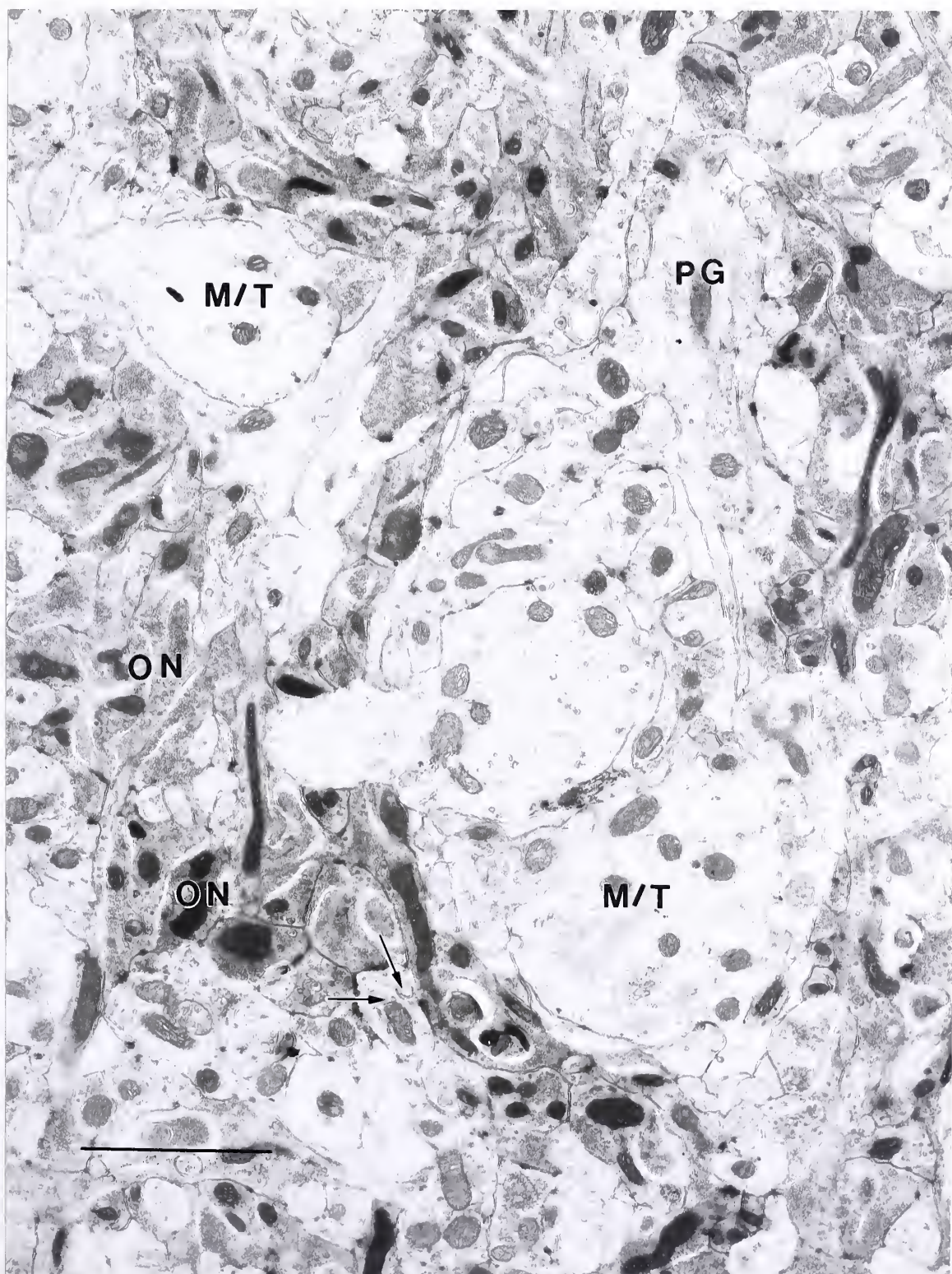


Figure 30. Electron micrograph taken within the glomerulus. Notice individual collection of islets of olfactory nerve terminals (ON) and paler dendritic processes. There were morphological differences among the dendritic processes. Large regularly shaped with intracytoplasmic organelles were identified as those belonging to mitral/tufted cells. Meanwhile, irregularly shaped and lightly flocculent ones corresponded to periglomerular cells. Arrows indicate direction or polarity of synapses. Abbreviations: M/T, mitral/tufted cell dendritic process; PG, periglomerular cell dendritic process; ON, olfactory nerve terminal islets. Scale bar= 2 $\mu$ m.









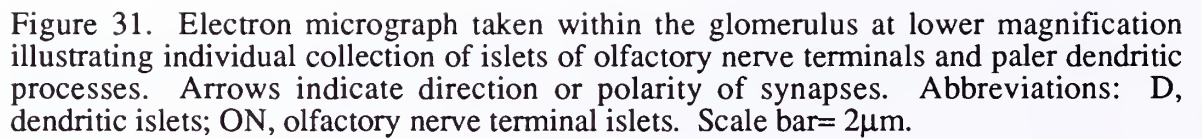
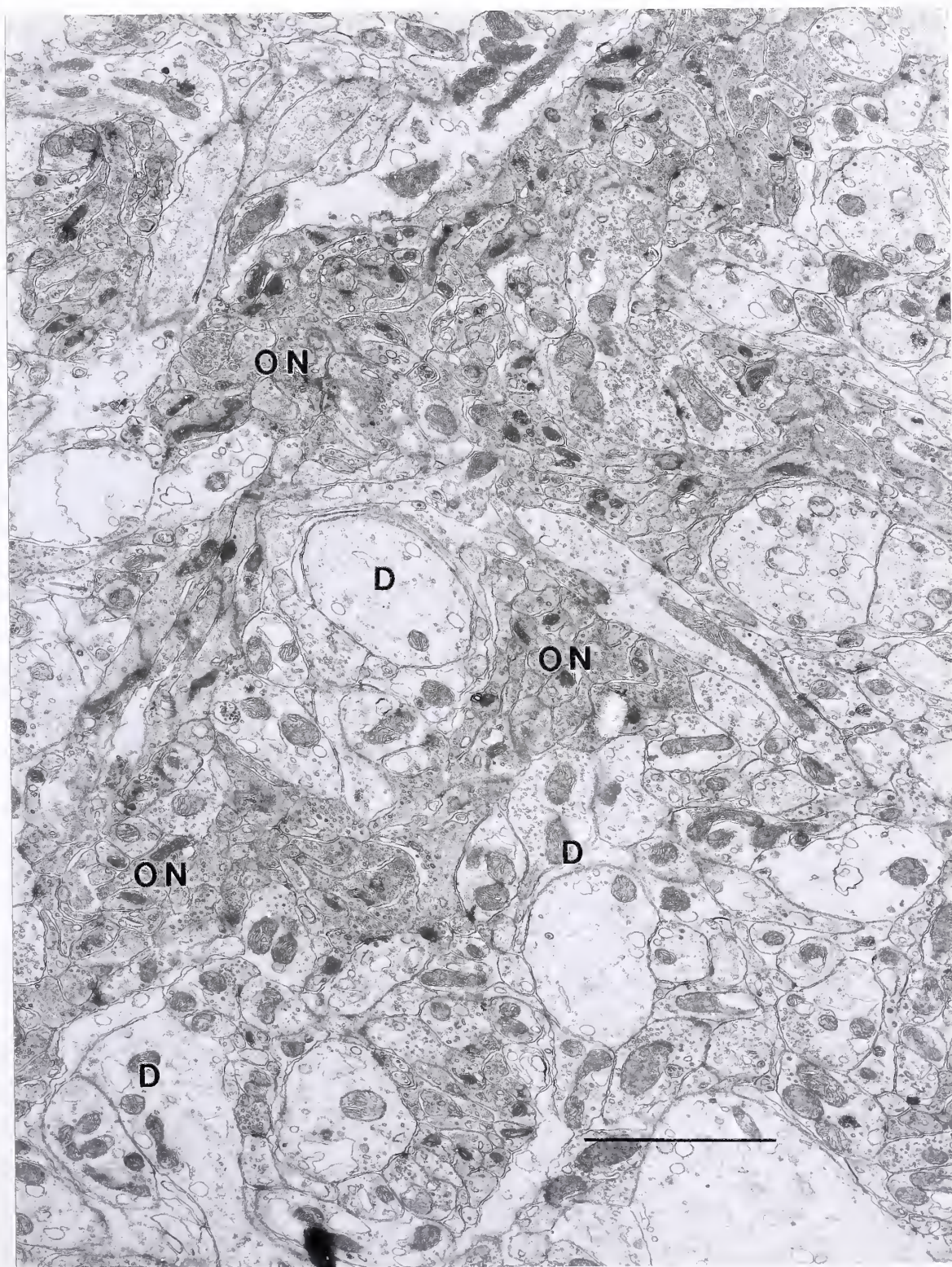
The image is a black and white electron micrograph showing a collection of small, dark, electron-dense structures (islets) of olfactory nerve terminals and paler, less dense dendritic processes. Arrows are visible, indicating the direction or polarity of synapses. The structures are clustered together, with some appearing more distinct than others. The background is light and grainy.

Figure 31. Electron micrograph taken within the glomerulus at lower magnification illustrating individual collection of islets of olfactory nerve terminals and paler dendritic processes. Arrows indicate direction or polarity of synapses. Abbreviations: D, dendritic islets; ON, olfactory nerve terminal islets. Scale bar= 2 $\mu$ m.









qualitative point of view it is prudent to state that there exists a difference in synaptic density between the shell and the core. In the shell many of the olfactory nerve terminals are seen establishing axodendritic synapses with mitral/tufted cell and periglomerular cells. In the core the incidence of islets containing olfactory nerve terminals was reduced, but also evident in the core were longitudinally cut olfactory nerve terminals (Figure 32) that rarely if at all made axodendritic contact. Dendrodendritic synapses were seen throughout the length of the montages. It remains to be established through a synaptological analysis if there is a difference in the density of type I and type II dendrodendritic synapses between the core and shell of a glomerulus. Based on preliminary observations in the deeper portions of the glomerulus, there seems to be a variable density of dendrodendritic synapses. It is in these area where most of the larger apical dendrites from mitral and tufted cells enter the glomerulus. The large dendritic size might account for a reduction in the number of type I synapses from mitral/tufted cell onto periglomerular cell dendrites. Again, it should be emphasized that this is from a qualitative point of view and more rigorous quantitative studies are needed.

## 2. Olfactory Nerve

Within the glomerulus olfactory axons travel for variable distances along which many varicosities can appear. The preterminal and terminal parts of the olfactory nerve are characteristically electron dense (Figure 33) compared to the axons found in the olfactory nerve layer (Figure 25). The darkening of these fibers occurs when they enter the glomerular structure and begin to loose their glial wrapping from ensheathing cells. As these fibers terminate they become very tortuous and extremely irregular in shape. The collection of axon terminals is always tightly packed and found, as mentioned earlier, in islets. There was never an instance where an isolated axon terminal was identified. On several occasions these islets resembled a whorl where the inner axons seemed to have a





Figure 32. Electron micrograph taken near the core of a glomerulus. Longitudinally cut sections of olfactory nerve axons (arrow) are found within this region among islets of olfactory nerve terminals and paler dendritic processes.. Notice lack of axodendritic synapses which is characteristic of these longitudinally oriented processes. Abbreviations: D, dendritic islets; ON, olfactory nerve terminal islets. Scale bar= 1 $\mu$ m.

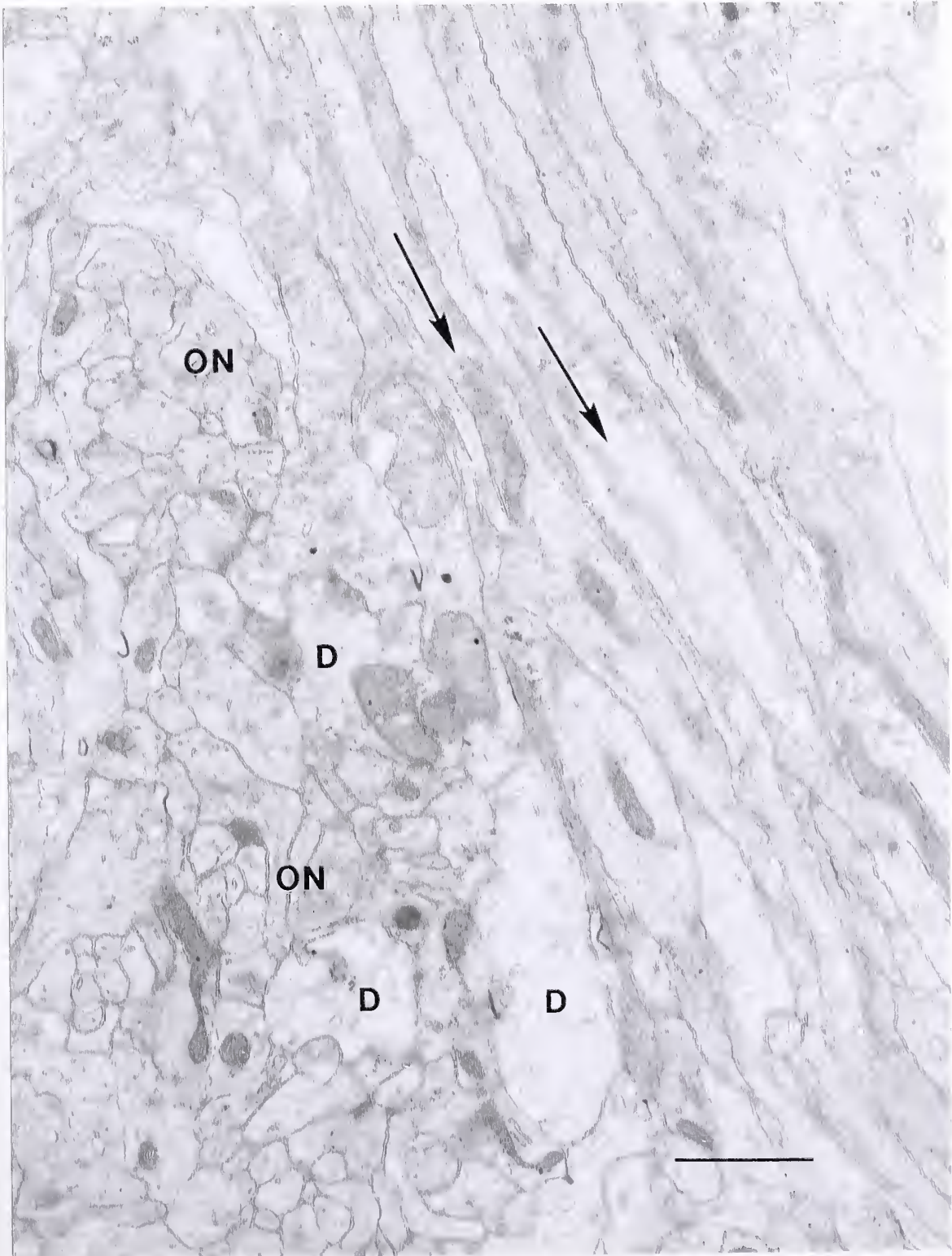
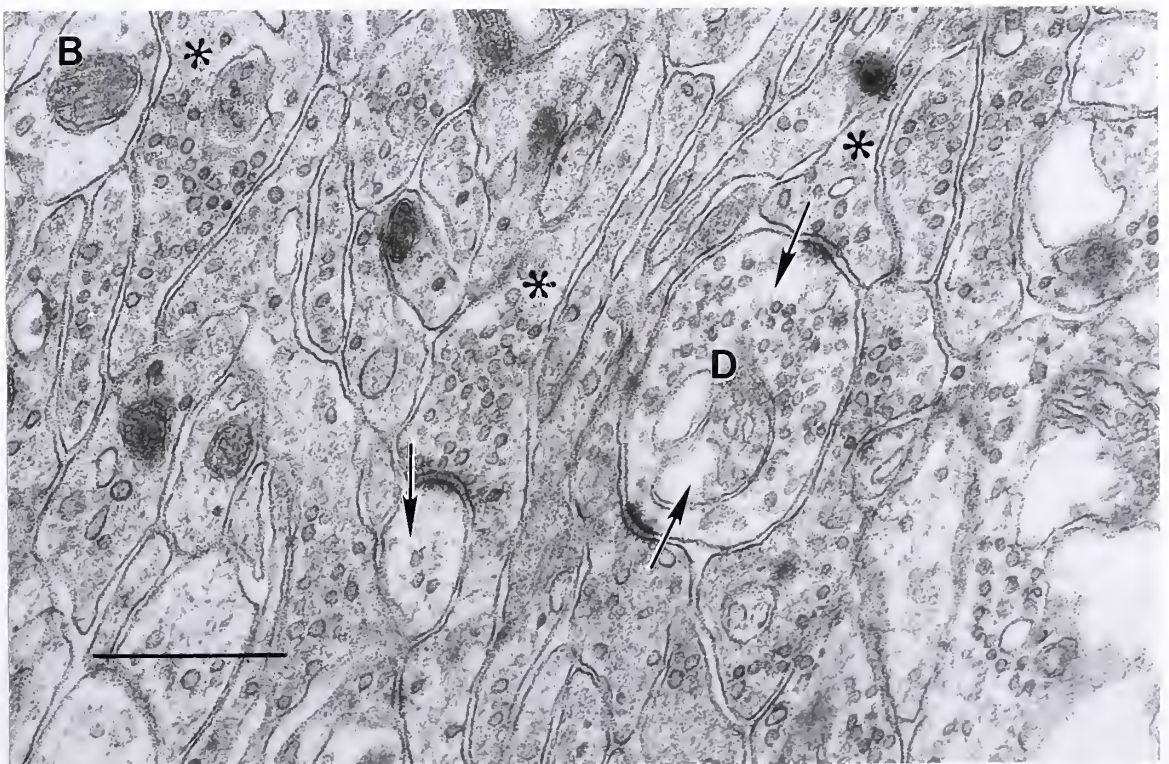
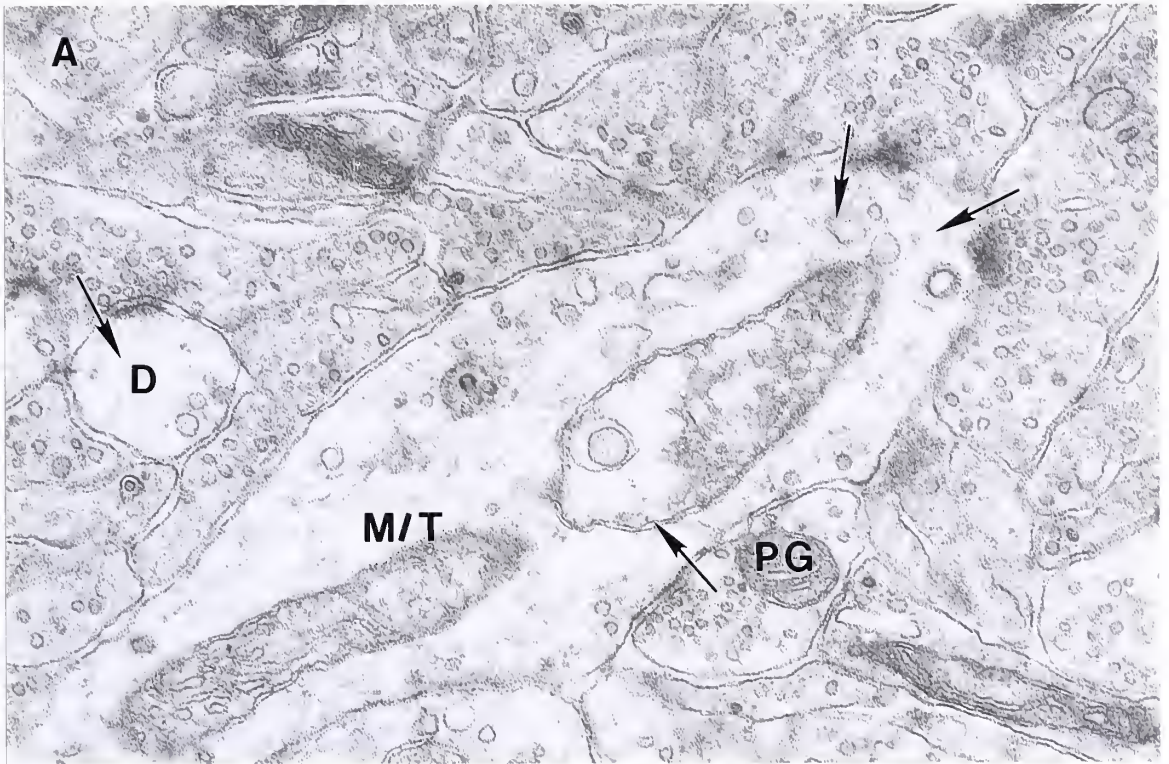








Figure 33. Electron micrograph illustrating axodendritic synapses. Olfactory nerve terminals are characteristically electron dense compared to dendritic profiles. The axon terminals are filled with spherical vesicles and make type I, asymmetrical synapse onto dendritic profiles. Also shown in (A) is an example of a type II, symmetrical synapse from a periglomerular cell dendritic process onto a presumed mitral/tufted cell terminal which receives a synapses from an olfactory nerve terminal. Arrows indicate direction or polarity of synapses and asterisks indicate olfactory nerve terminals. Abbreviations: D, dendritic processes; PG, periglomerular cell dendritic process; M/T, mitral tufted/cell dendritic process. Scale bar= 0.5 $\mu$ m.







better defined shape compared to the more irregular axons located on the outside. Usually the outside axon terminals were involved in establishing axodendritic synapse.

The preterminal and terminal *en passant* boutons have a dark, granular and vesicle filled axoplasm. Small mitochondria and cistern-like organelles were usually present on these terminals. The groups of terminals were separated by a thin gap of extracellular space. The olfactory axon terminals were always seen making type I asymmetrical synapses onto paler, irregularly shaped dendritic processes (Figure 33). These synapses were characterized by a dense collection of spherical vesicles closely juxtaposed to a postsynaptic dendrite. Within the postsynaptic dendrite, a dense postsynaptic membrane extended into the cytoplasm. Individual olfactory nerve terminals were seen making more than one synapse onto several dendritic processes. In the same manner, several olfactory nerve terminals were seen making synapses onto one dendritic process. The olfactory nerve terminals terminated predominantly onto finer smaller size dendritic processes, although few made contact with large processes. There was no evidence of an olfactory nerve terminal receiving any synaptic contact from other axon terminals or dendritic processes. There were however tight junctions between axonal processes especially when three or more axon terminals came in close contact. This junction characteristically included a dense membrane within the axon that should not be confused with a synapse and that Berger (1969) has described as desmosomes.

### 3. Mitral/Tufted and Periglomerular Cell Dendritic Processes

At the level of the light microscope the dendritic processes of mitral and tufted cells differ in their distribution and amount of branching (Pinching and Powell, 1971a). At the level of the electron microscope it is virtually impossible to distinguish between them. Both cell types exhibit large dendritic profiles and receive and establish synaptic contact with the same processes in the same manner. Therefore, they will be considered together, as has been traditionally in studies of the olfactory bulb (Shepherd and Greer,



1990). Also important to note at this juncture is that the identification between periglomerular vs. mitral/tufted cell dendrites relies to an extent on a previous observation that employed serial reconstructions (Pinching and Powell, 1971b).

The mitral/tufted cell dendrites were consistently pale and found within the glomerulus cut at different angles and at all stages of branching. The dendritic processes had consistently a regular outline, usually circular when cut cross sectionally, and were of varying sizes. When cut longitudinally the mitral/tufted cell dendrites had a cylindrical shape with even outlines and varying sizes. Within the cytoplasm organelles such as endoplasmic reticulum, ribosomes, mitochondrion and microtubules were evident. Large dendritic profiles of this type were usually seen in close approximation to the cell bodies in the periglomerular region (D in Figures 27 and 29). This might represent the proximal segments of the mitral/tufted cell apical dendrites before they arborize in the glomerulus.

The dendritic processes of mitral/tufted cell received type I, symmetrical synapses from olfactory nerve terminals. For the most part these synapses were made on medium to small size processes, although some were observed on very large longitudinally cut dendritic processes. In several instances, one mitral/tufted dendrite received multiple synapses from different olfactory nerve terminals. The presence of dispersed spherical vesicles was apparent throughout the cytoplasm of mitral/tufted cell dendritic processes. However, in the majority of cases these vesicles were clustered together and forming a type I, asymmetrical synapses onto presumed periglomerular cell dendritic processes (Figure 34). On very few occasions a reciprocal type II, symmetrical synapse (Figure 36) was made from the periglomerular process onto the mitral/tufted cell dendrite process. No instance was found where an identified mitral/tufted cell profile made a type I synapses onto a dendritic profile that either received a type II synapse or made a type I synapse. This suggests that mitral/tufted cell dendrites do not interact with each other monosynaptically.







Figure 34. Electron micrograph illustrating dendrodendritic synapses. Large pale presynaptic dendritic processes identified as mitral/tufted cell dendrites establish type I, asymmetrical synapses onto presumed periglomerular cell dendritic processes. Arrows indicate direction or polarity of synapses. Abbreviations: PG, periglomerular cell dendritic process; M/T, mitral/tufted cell dendritic process. Scale bar= 0.5 $\mu$ m.

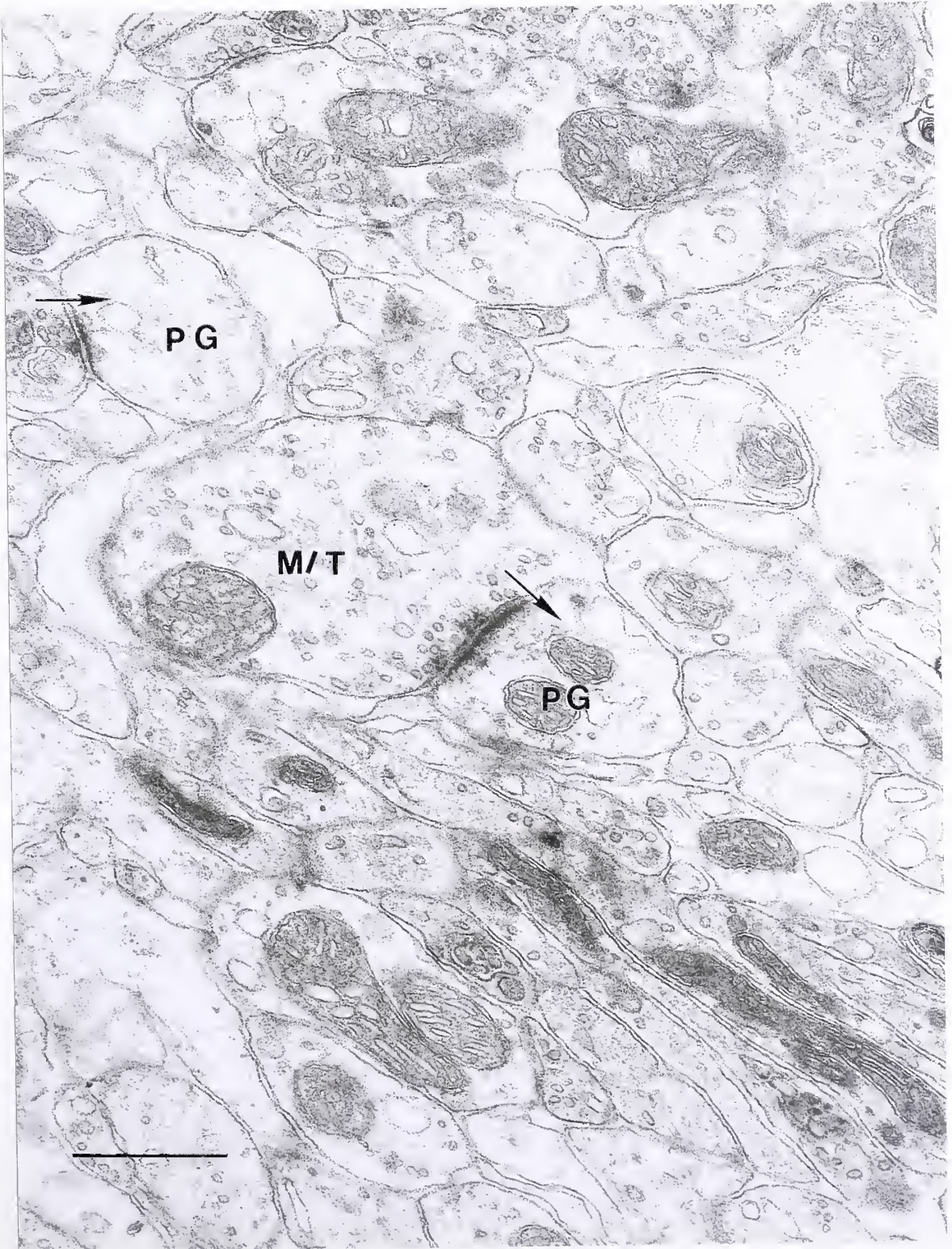






Figure 35. Electron micrograph illustrating type II, dendrodendritic synapses. Dendritic processes identified as periglomerular cell dendrites make type II, symmetrical synapses onto mitral/tufted cell dendritic processes. In A a single mitral/tufted cell receives two symmetrical synapses from two periglomerular cell processes. Notice the presence of flattened vesicles within the cytoplasm of periglomerular cell dendritic processes. In B a periglomerular cell process makes a symmetrical synapse onto a mitral/tufted cell dendrite. Notice the characteristically flocculent cytoplasm and flattened vesicles of the two periglomerular dendritic process compared to the mitral/tufted cell dendrites. Arrows indicate direction or polarity of synapses. Abbreviations: PG, periglomerular cell dendritic process; M/T, mitral/tufted cell dendritic process. Scale bar= 0.5 $\mu$ m.



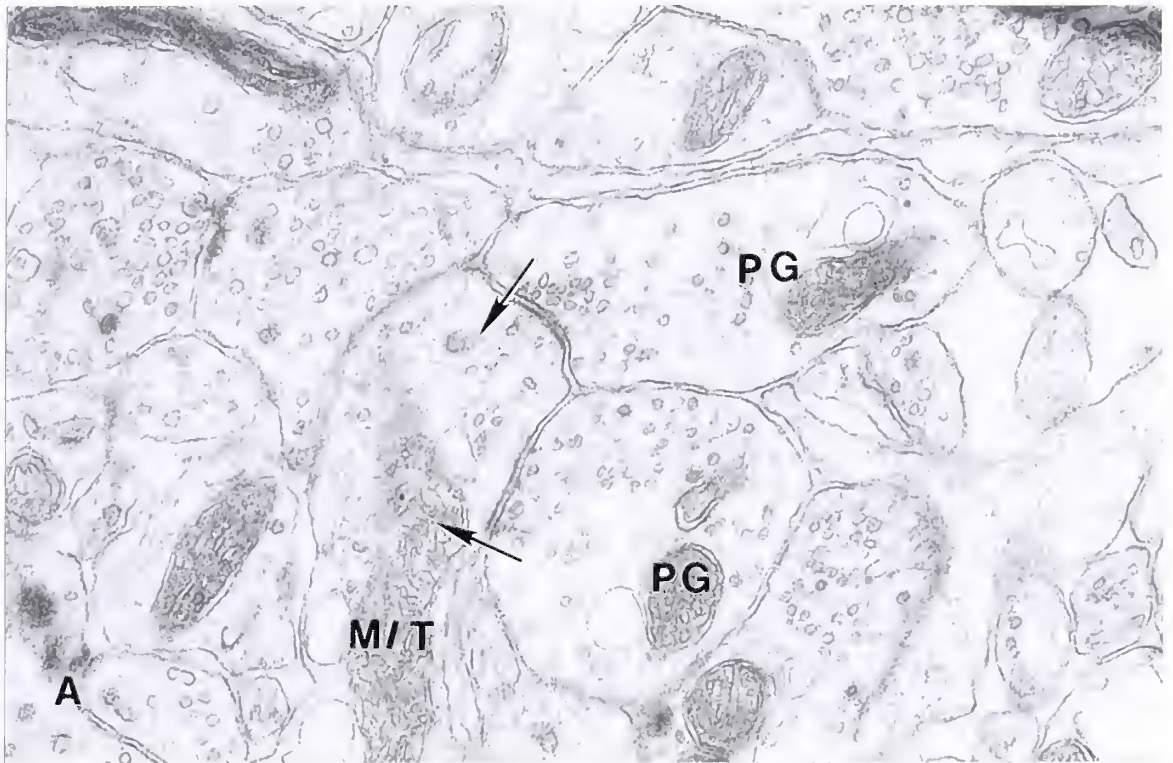
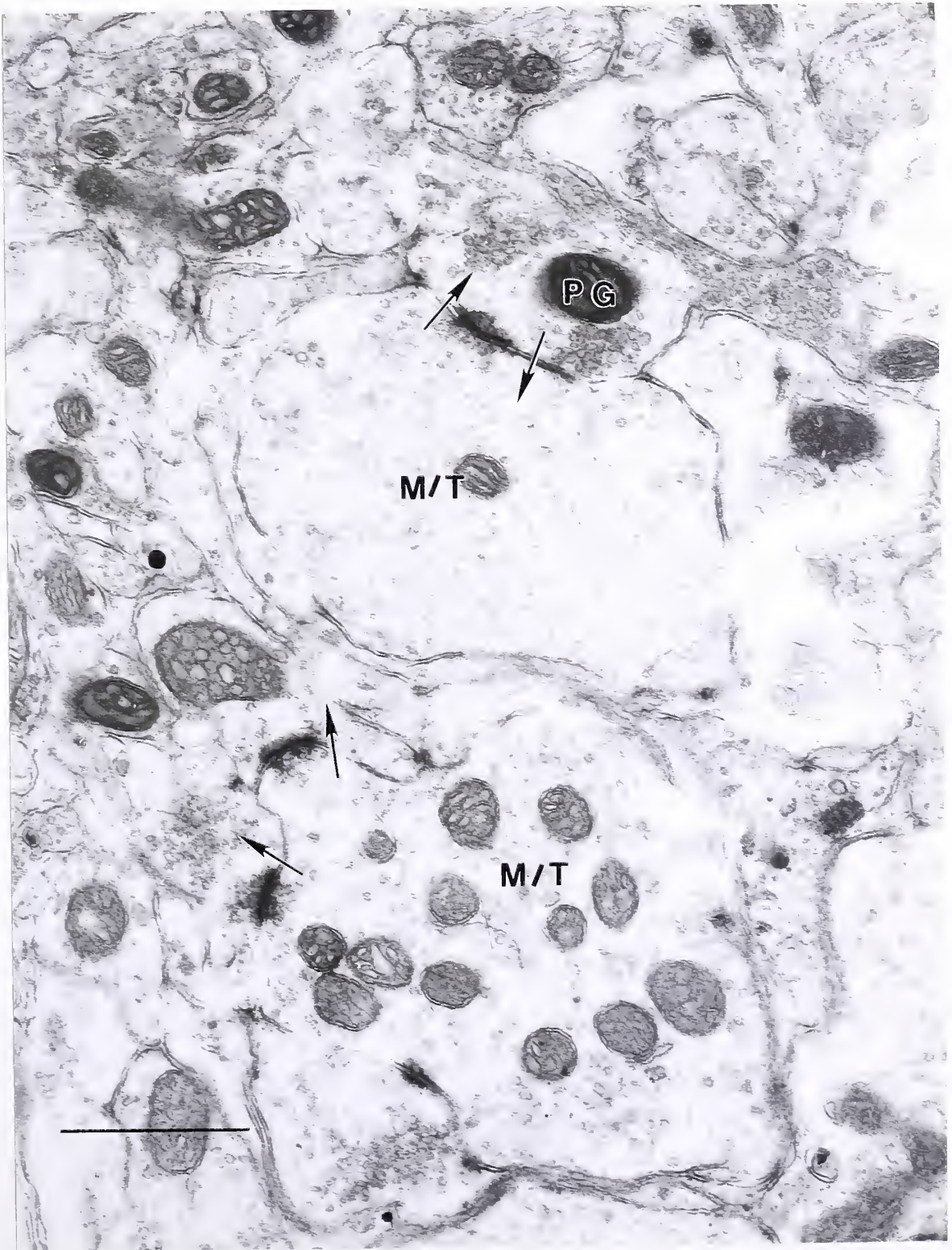








Figure 36. Electron micrograph illustrating reciprocal synapses. In the upper sections of figure a mitral/tufted cell dendritic process makes a type I asymmetrical synapse to a periglomerular cell dendritic process. Immediately adjacent a type II, symmetrical synapse is made from the periglomerular cell dendritic process. In the lower portion of figure a mitral/tufted cell dendritic process makes multiple synaptic contacts with several periglomerular cell processes. Arrows indicate direction or polarity of synapses. Abbreviations: PG, periglomerular cell dendritic process; M/T, mitral/tufted cell dendritic process. Scale bar= 1 $\mu$ m.





Periglomerular cell dendritic processes exhibited a very tortuous and irregular outline as compared to mitral/tufted cell dendrites (PG in Figure 30). Intracytoplasmic organelles such as endoplasmic reticulum, ribosomes, free or arranged in rosettes, mitochondria, and microtubules were present in periglomerular cell dendritic processes. One distinguishing characteristic found in periglomerular cells was the greater abundance of flattened vesicles within the cytoplasm, as compared to mitral/tufted cell dendritic processes. In some cases there were extensive cisternae of endoplasmic reticulum with numerous ribosomes. The combination of numerous vesicles and the presence of endoplasmic reticulum often gave the periglomerular cell processes a darker more granular appearance.

The periglomerular cell processes received type I, asymmetrical synapses from olfactory nerve terminals and mitral/tufted cell processes. Periglomerular cell processes made type II, symmetrical synapses onto mitral/tufted cell dendritic processes (Figure 35). These synapses were characterized by a collection of flattened, pleomorphic vesicles juxtaposed to a symmetrical postsynaptic membrane specialization. As mentioned above, on few occasions the periglomerular cell processes were involved in reciprocal synapses with mitral/tufted cell dendritic processes. There was no instance where an identified periglomerular process was seen making a type II synapse onto a process that received a type I synapse from a mitral/tufted cell dendrite. This excludes the notion that periglomerular cell synaptically interact with each other within the glomerulus via dendritic specializations.

## **E. SUMMARY**

The observations described above are consistent with those presented in previous studies (Pinching and Powell, 1971a,b; White, 1972, 1973). The olfactory nerve axons usually enter the glomerulus from the superficial aspect. Within the glomerulus these terminals become electron dense containing many spherical vesicles within their







axoplasm. Mitral and tufted cell processes have an electron lucent cytoplasm with several organelles such as mitochondria and smooth endoplasmic reticulum present and arrays of microtubules distributed throughout the dendrites. The periglomerular cell processes have an electron dense cytoplasm but share a number of similarities with the mitral and tufted cells such as mitochondria and smooth endoplasmic reticulum. In addition to these morphological characteristics the polarity or direction of synapses between these processes, for example the olfactory nerve making a type I, asymmetrical synapse with either periglomerular or mitral/tufted cells or the reciprocal synapse between the latter two, facilitated the identification of these processes in our analyses. The functional properties of these synapses were mentioned above. In brief, olfactory nerve terminals make excitatory synapses, onto mitral/tufted and periglomerular cell dendrites. Recent studies suggest that the neurotransmitter responsible for this excitation is glutamate (Berkowicz et al., 1993; Sassoe et al., 1993). Similarly, mitral/tufted cell dendrites make excitatory synapses onto periglomerular cell processes and these in turn reciprocate with an inhibitory synapse. In the current study it was very rare that a reciprocal synapse, that is adjacent excitatory and inhibitory synapses, was seen in a single photomicrograph. We encountered most commonly individual excitatory and inhibitory synapses among dendrodendritic synapses.

The overall anatomical organization of the glomerulus suggested a degree of subcompartmentalization composed of islets of axon terminals and dendritic processes. It seemed that the synaptic density was differentially organized as well. Most of the axodendritic synapses were made onto medium to small caliber size dendritic profiles. Likewise, dendrodendritic synapses were predominantly involved in medium to small size dendritic profiles. This heterogeneous organizational pattern confers the possibility that within individual glomeruli there exists a subglomerular organization that might subserve differential, possibly parallel, pathways for odor processing.



#### **IV. STUDY III-ELECTRON MICROSCOPY IMMUNOCYTOCHEMISTRY**

##### **A. PURPOSE OF STUDY**

Comparatively little is known regarding the synaptic organization of the olfactory bulb glomerulus. It continues to remain unclear if there is a homogeneous synaptic distribution and organization among periglomerular cell and other subpopulation of neurons contributing processes to the glomerulus. The possibility that different receptor cell populations may preferentially synapse with either projection or interneurons has not been addressed. This study will attempt to characterize the synaptic organization of the olfactory bulb glomerulus by comparing the synaptic organization of subpopulations of periglomerular cells immunoreactive for tyrosine hydroxylase and GABA. Based on anatomical, physiological and immunocytochemical observations, it is hypothesized that there is a heterogeneous or differential synaptic distribution among subpopulations of periglomerular and projection neurons.

##### **B. TISSUE COLLECTION**

Four adult Sprague-Dawley rats (two females, two males) weighing 154-200 grams were euthanized and perfused in a similar fashion as outlined in Study I. The perfusate consisted of 4% paraformaldehyde and 0.2% glutaraldehyde. Through several trials the 0.2% glutaraldehyde concentration was empirically found to provide the best tissue preservation with adequate primary antibody staining. Following perfusion the brains were removed and processed similarly to Study I.

##### **C. STAINING PROCEDURE**

The free floating sections were washed in amino acid supplement (AAS) consisting of 1.0% Bovine Albumin, 0.1% Glycine and 0.1% Lysine in 0.1M PB 5X 7 minutes each. Following the wash, the sections were then preincubated in 5% normal



goat serum and 1% bovine serum in 0.1M PB for those sections determined to be stained for tyrosine hydroxylase and GABA respectively. Following preincubation the sections were washed 2X 15 minutes in 0.1M PB and then incubated for 48 hours at 4°C in primary antibody diluted in AAS to 1:1200 and 1:1000 for tyrosine hydroxylase and GABA respectively. The primary antibody against GABA was raised in guinea-pig and for tyrosine hydroxylase the antibody was raised in rabbit.

Following incubation the sections were rinsed in 0.1M PB 5X 10 minutes each and then incubated in secondary antibody for 90 minutes. The secondary antibody consisted of biotinalated goat anti guinea-pig IgG (1:200, Vector Labs.) and biotinalated anti rabbit IgG (1:400, Vector Labs.) for GABA and tyrosine hydroxylase respectively. After the incubation period the sections were washed 5X 10 minutes each with 0.1M PB. Thirty minutes prior to the last wash the avidin-biotin-complex peroxidase (Vectastain Elite ABC kit) was prepared (2 drops of avidin and two drops of biotin mixed in 10 ml of 0.1M PB). The sections were incubated for 45 minutes with the avidin-biotin-complex peroxidase followed by 5X 7 minutes washes, of Tris buffer.

Following the rinse the tissue was incubated between 60 to 90 seconds in a solution consisting of 0.1% 3,3'-diaminobenzidine tetrachloride dehydrate (DAB) and 0.3% Hydrogen peroxidase. After the incubation period the sections were rinsed in Tris buffer 5X 7 minutes each.

For electron microscopy preparation the sections were placed in a vial containing 2% osmium tetroxide in 0.1M PB and rotated for 1 hour. Subsequently the tissue was placed in 50% Ethyl Alcohol (ETOH) for ten minutes followed by ten minutes in 70% ETOH. The sections were then placed in 1% uranyl acetate (0.0626gms of uranyl acetate in 6.26 ml of 70% ETOH) for 1 hour in the dark. Following the uranyl acetate procedure the sections were routinely dehydrated through graded alcohols (10 minutes 70%, 20 minutes in 90% and 45 minutes in 100%) and cleared in 2X 5 minute baths of xylene.





The dehydrated sections were placed in a vile containing a 1:1 solution of xylene and Epon and left in a rotator overnight.

The next day the sections were embedded in Epon and placed on a slide. The slides containing the tissue were placed in an oven at 60° C for 48 hours. The embedded tissue was examined under the light microscope and small sections that contained several glomeruli with stained cell bodies were dissected, mounted onto an Epon block and placed in the oven at 60° C for 36- 48 hours. During dissection careful attention was taken to exclude superficial tufted cells immunoreactive for tyrosine hydroxylase. The blocks were removed from the oven and thick, 1µm, sections containing glomeruli were stained with Toluidine Blue and utilized for orientation prior to and during thin sectioning. Thin silver sections, approximately 70nm, were cut on a Reichert Ultracut E microtome and mounted on Formvar coated 1 X 2 mm slot grids. The grids were lightly stained with lead citrate (Reynolds et al., 1961) and examined in a JEOL 1200 electron microscope at 80kV aperture of 2 and photographed with an exposure of 2 mamp/sec.

#### **D. DATA AQUISITION**

Photomicrographs within individual glomeruli of stained and unstained processes were taken at a primary magnification of X15,000 to 20,000. The photomicrographs were printed on Kodak P2 paper for a final printed magnification of X45,000 to 60,000. Based on previous observations we were able to identify the cellular constituents that form individual glomeruli. Based on previous observations on Study-II and the synaptic organization we were able to identify individual cellular constituents that form individual glomeruli such as olfactory nerve, mitral/tufted cell and periglomerular cell dendrites. Mitral and tufted cell dendrites having undergone their primary branching within the glomeruli appeared virtually indistinguishable from each other. Therefore, no attempt was made to differentiate between them.





For each micrograph all recognizable stained and unstained processes were labeled and identified as either olfactory nerve terminal, periglomerular or mitral/tufted cell processes or unknown. A synapse was defined as containing a clearly defined post synaptic specialization together with one or more vesicles juxtaposed to the presynaptic specialization. Following identification each printed micrograph was scanned using an Apple scanner and saved in an Macintosh Quadra for further analysis.

## **E. DATA ANALYSIS**

Image 1.49, courtesy of NIH, was utilized to perform all the quantitative measurements for each identified cellular process excluding olfactory nerve terminals. Such measurements included, dendritic circumference, cross sectional size measured at its longest axis, and cross sectional area. Synaptological analyses included polarity of synapses, synaptic frequency, symmetry, and geometry (planar, convex and concave) in relation to the juxtaposed post-synaptic profile of synaptic terminals. The quantitative measurements were statistically analyzed and stored using Excel 4.0.

## **F. RESULTS**

### **1. Tyrosine Hydroxylase**

At the electron microscopic level immunoreactive processes were easily recognized for both GABA and tyrosine hydroxylase. Tyrosine hydroxylase immunoreactive processes were identified by their stippled, granular and dark appearance (THIR in Figure 37 A and B). These processes often contain cytoplasmic organelles such as mitochondria (asterisk in Figure 37 A). The olfactory nerve (ON in Figure 37 B) terminals were easily recognized by their darker appearance and vesicle filled axoplasm. Also present were several large pale profiles suggestive of mitral/tufted cell processes cut longitudinally (M/TL in Figure 37 B) or cross sectionally (M/TC in Figure 37 B). These



processes were identified as dendritic based on their irregular shape and intracytoplasmic organelles such as mitochondria and microtubules (arrow head in Figure 37 B).

Cell bodies immunoreactive to tyrosine hydroxylase (Figure 38) were identified in the periphery of individual glomeruli. These cell bodies had a characteristic darker staining compared to surrounding cell bodies (Figure 38 A). These cell bodies were identified as periglomerular cells based on their morphological characteristics which include small size, a thin rim of cytoplasm surrounding individual nuclei (between arrow heads in Figure 38), and intranuclear invaginations (arrows in Figure 38). Olfactory receptor axon terminals established type I excitatory synapses onto mitral/tufted cells and tyrosine hydroxylase immunoreactive dendritic processes (Figure 39). Notice the morphological characteristics of these synapses with round presynaptic vesicles and an asymmetrical postsynaptic specialization. Also found in Figure 39 is the only illustration encountered where one olfactory nerve terminal (asterisk in Figure 39) establishes synaptic contact with a mitral/tufted dendrite while simultaneously making another synapse with an immunoreactive process. This pattern of connectivity suggests divergence of odor information from a single olfactory axon. Many instances were seen when an individual mitral/tufted dendritic processes received multiple synapses from several olfactory nerve terminals while an immunoreactive processes received a single synapse from one olfactory nerve terminal. Although not illustrated, darker profiles with intracytoplasmic organelles presumably identified as non-immunoreactive periglomerular cells received synaptic contact from olfactory nerve terminals. No conventional synapses were observed between olfactory nerve terminals or from dendritic processes onto olfactory nerve terminals .

The majority of synapses observed involving tyrosine hydroxylase immunoreactive processes were dendrodendritic (Figure 40). Large pale profiles, presumably mitral/tufted cell dendrites, established type I, excitatory synapses onto immunoreactive processes. Although the immunoreactive processes had a very darkly





Figure 37. Electron micrograph of rat olfactory bulb immunohistochemically stained using anti-tyrosine hydroxylase primary antibodies. (A) Notice darkly stained immunoreactive profile (THIR) compared to vesicle filled axoplasm of olfactory nerve axon terminals (ON). Arrows indicated polarity or directions of synapses in this example all synapses are axodendritic type I synapses characterized by an asymmetrical membrane specialization. (B) Immunoreactive profiles were easily identified. Also evident is a longitudinally and cross sectionally cut mitral/tufted cell dendritic process (M/TL and M/TC respectively). Dendritic processes were identified based on their large and irregular shaped and by the presence of intracytoplasmic organelles such as mitochondrion (asterisk) and microtubules (arrow heads). Arrows indicated polarity or directions of synapses. Abbreviations: ON, olfactory nerve terminals; M/T, mitral/tufted dendritic process; THIR, tyrosine hydroxylase immunoreactive process. Scale bar= 1 $\mu$ m.



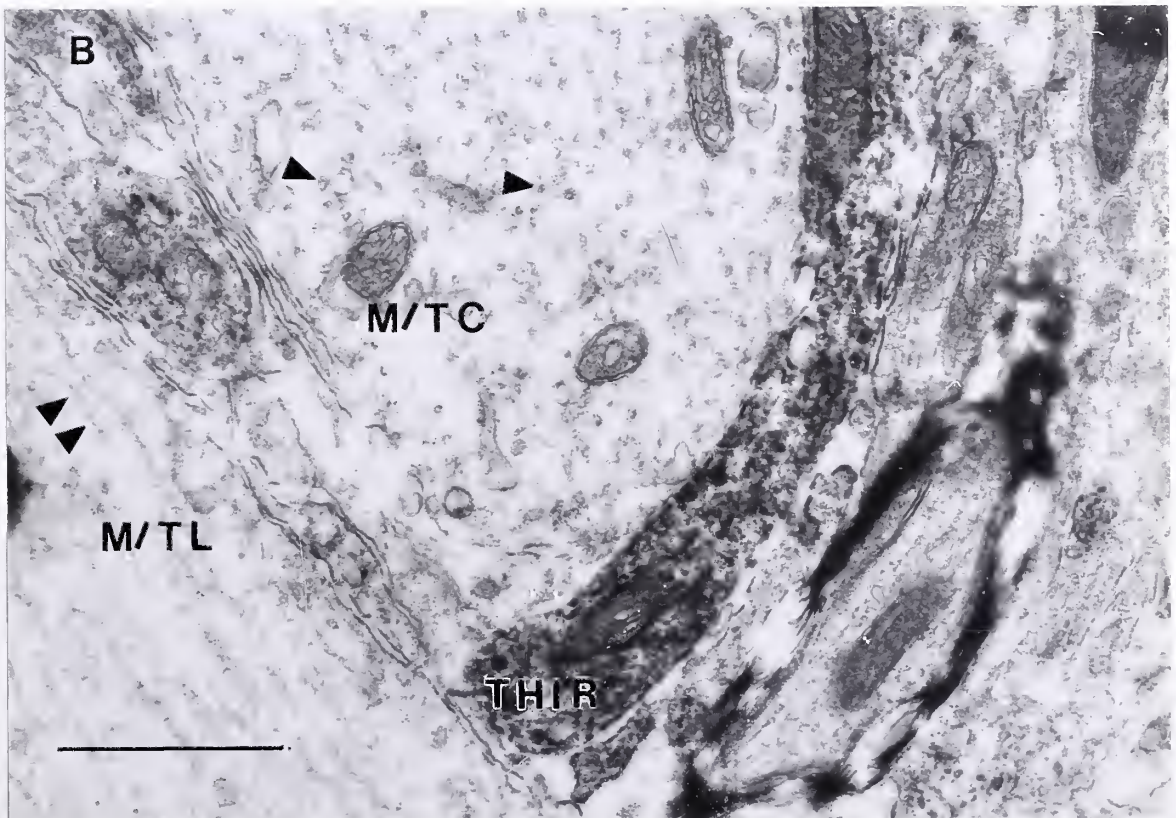






Figure 38. Electron micrograph of rat olfactory bulb immunohistochemically stained using anti-tyrosine hydroxylase primary antibodies. Periglomerular cell bodies immunoreactive and non-immunoreactive for tyrosine hydroxylase. A and B, immunoreactive cell body is evident by its characteristic darkly stained nucleus and cytoplasm. These cells were identified as periglomerular cells based on their small size, thin rim of cytoplasm surrounding nucleus (between arrow heads), and intranuclear invaginations (arrows). Abbreviation: NUC, nucleus; PG, periglomerular cell; THIR, tyrosine hydroxylase immunoreactive. Scale bar in A= 2 $\mu$ m and in B= 1 $\mu$ m.



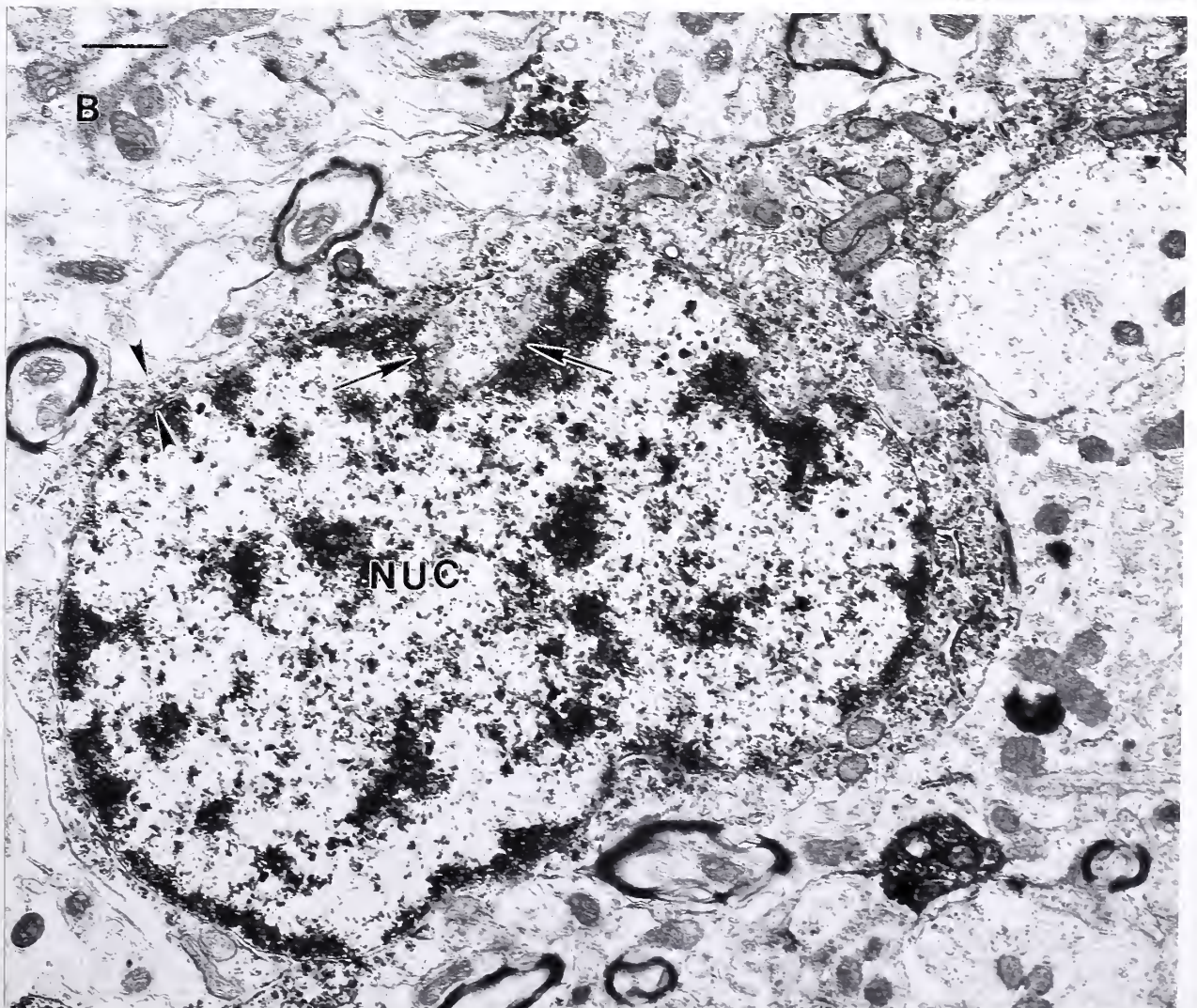










Figure 39. Electron micrograph of rat olfactory bulb immunohistochemically stained using anti-tyrosine hydroxylase primary antibodies. Illustration of olfactory nerve terminals making type I synapses onto tyrosine hydroxylase and mitral/tufted cell dendritic process. Notice that a single olfactory nerve terminal (asterisk) is simultaneously making type I, excitatory synapses onto a mitral/tufted and a tyrosine hydroxylase immunoreactive processes. Often mitral/tufted cell dendritic processes received synapses from multiple olfactory nerve terminals. Arrows indicated polarity or directions of synapses. Abbreviations, ON, olfactory nerve terminals; M/T, mitral/tufted dendritic process; THIR, tyrosine hydroxylase immunoreactive process. Scale bar= 1 $\mu$ m.

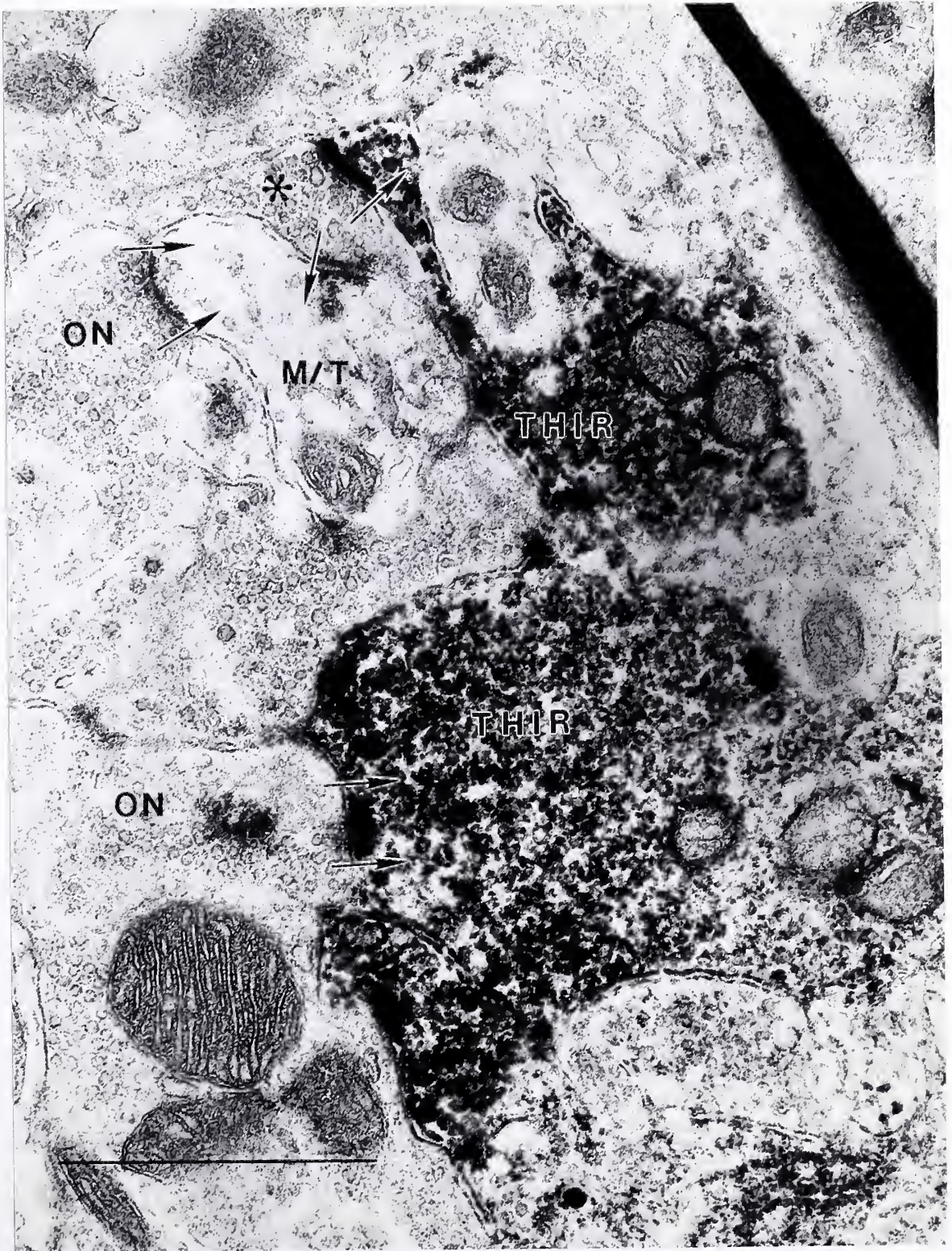
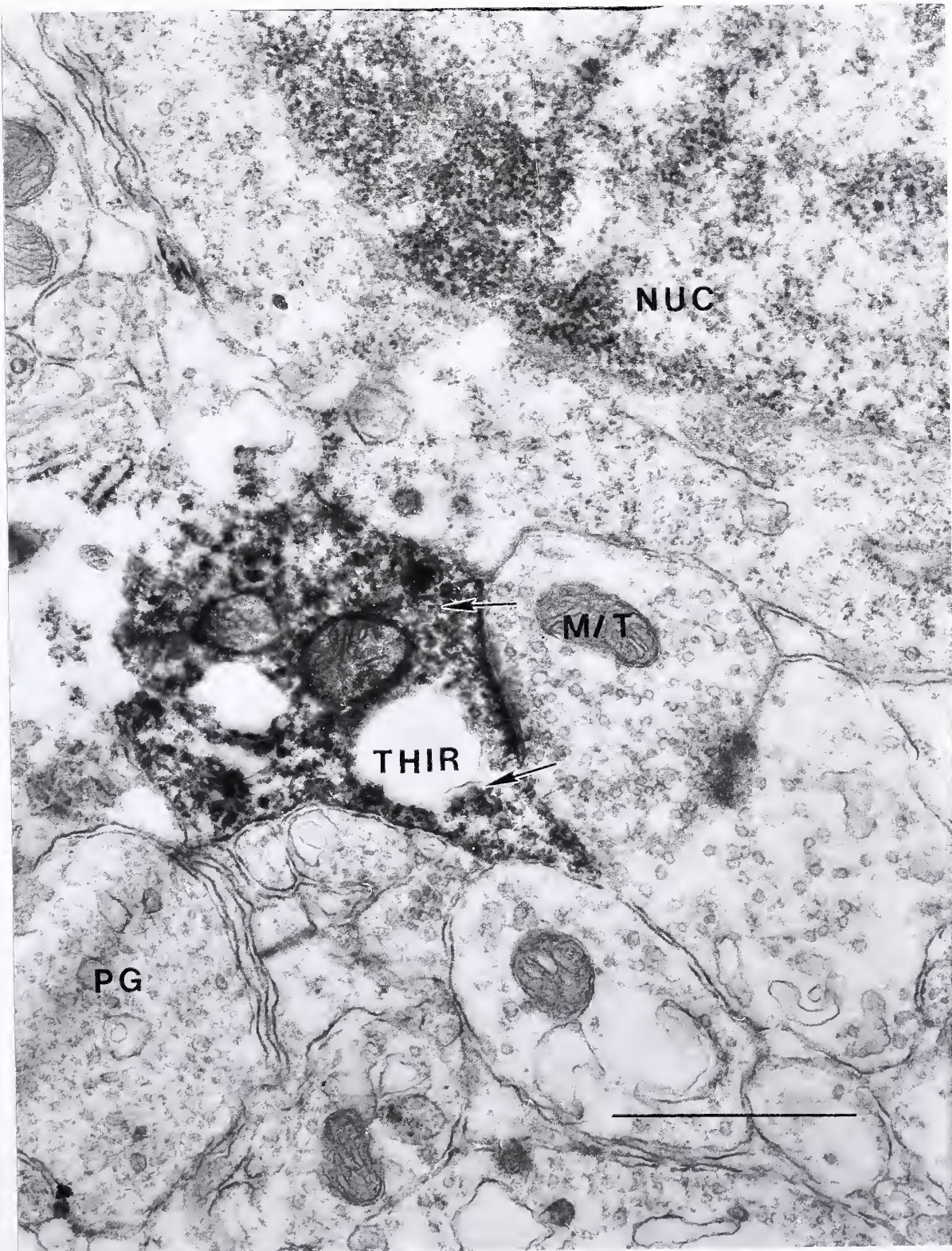








Figure 40. Electron micrograph of rat olfactory bulb immunohistochemically stained using anti-tyrosine hydroxylase primary antibodies. Illustration of dendrodendritic synaptic interaction between a mitral/tufted dendritic process and a tyrosine hydroxylase immunoreactive process. Notice the type I synapse with its morphological characteristics such as round vesicles and an asymmetric postsynaptic membrane specialization. Also illustrated is periglomerular cell dendritic process. This process has a darker cytoplasm compared with a mitral/tufted cell process. Arrows indicated polarity or directions of synapses. Abbreviations: M/T, mitral/tufted dendritic process; THIR, tyrosine hydroxylase immunoreactive process; PG, periglomerular cell dendritic process; NUC, nucleus. Scale bar= 1 $\mu$ m.







stained profile, this did not impede visualizing a postsynaptic specialization. The presence of vesicles within the presynaptic dendritic profiles aided in assessing the presence of a synapse. Periglomerular cell processes were clearly identified as having a darker appearance from the mitral/tufted cell dendritic processes. These processes also received type I synapses from mitral/tufted cell dendritic processes (not shown).

Tyrosine hydroxylase immunoreactive processes established dendrodendritic type II, inhibitory synapses onto mitral/tufted cell dendritic processes (Figure 41). It is important to notice the differences in the postsynaptic specializations found between type I and type II synapses. In the upper one third of Figure 41, an axon terminal is making an asymmetrical (type I) synapse onto a mitral/tufted dendrite evident by its thicker postsynaptic membrane specialization. Simultaneously, an immunoreactive process makes a symmetrical (type II) synapse onto the same mitral/tufted dendrite with a thinner post synaptic membrane specialization. Figure 42 shows another example of a tyrosine hydroxylase immunoreactive process making a type II, inhibitory synapse onto a longitudinally cut mitral/tufted cell process. Not all type II synapses were made by immunoreactive processes. In Figure 43 a presumed periglomerular cell process is making a symmetrical type II synapse onto a mitral/tufted cell process in the vicinity of an immunoreactive process. In turn the mitral/tufted cell process is making an asymmetrical type I synapse onto a tyrosine hydroxylase immunoreactive process. So this forms, collectively, a circuit where the periglomerular cell process presumably inhibits the mitral/tufted cell process preventing the release of the excitatory vesicles that would consequentially excite the immunoreactive cell. There was no evidence of synaptic interaction between two immunoreactive processes. Likewise there was no evidence of synaptic interaction between two periglomerular cell dendrites or between a tyrosine hydroxylase immunoreactive and a periglomerular cell dendrite. Immunoreactive processes made type II synapses only. There was no evidence of an immunoreactive





Figure 41. Electron micrograph of rat olfactory bulb immunohistochemically stained using anti-tyrosine hydroxylase primary antibodies. Tyrosine hydroxylase immunoreactive processes make type II, symmetrical synapses onto mitral/tufted cell dendritic processes. Also shown is a type I, asymmetrical synapse from an olfactory nerve terminal onto a mitral/tufted cell dendritic process. Notice the difference in the postsynaptic membrane specialization thickness between both synapses. Arrows indicate direction or polarity of synapse. Abbreviations ON, olfactory nerve terminals; M/T, mitral/tufted dendritic process; THIR, tyrosine hydroxylase immunoreactive process. Scale bar= 1 $\mu$ m.



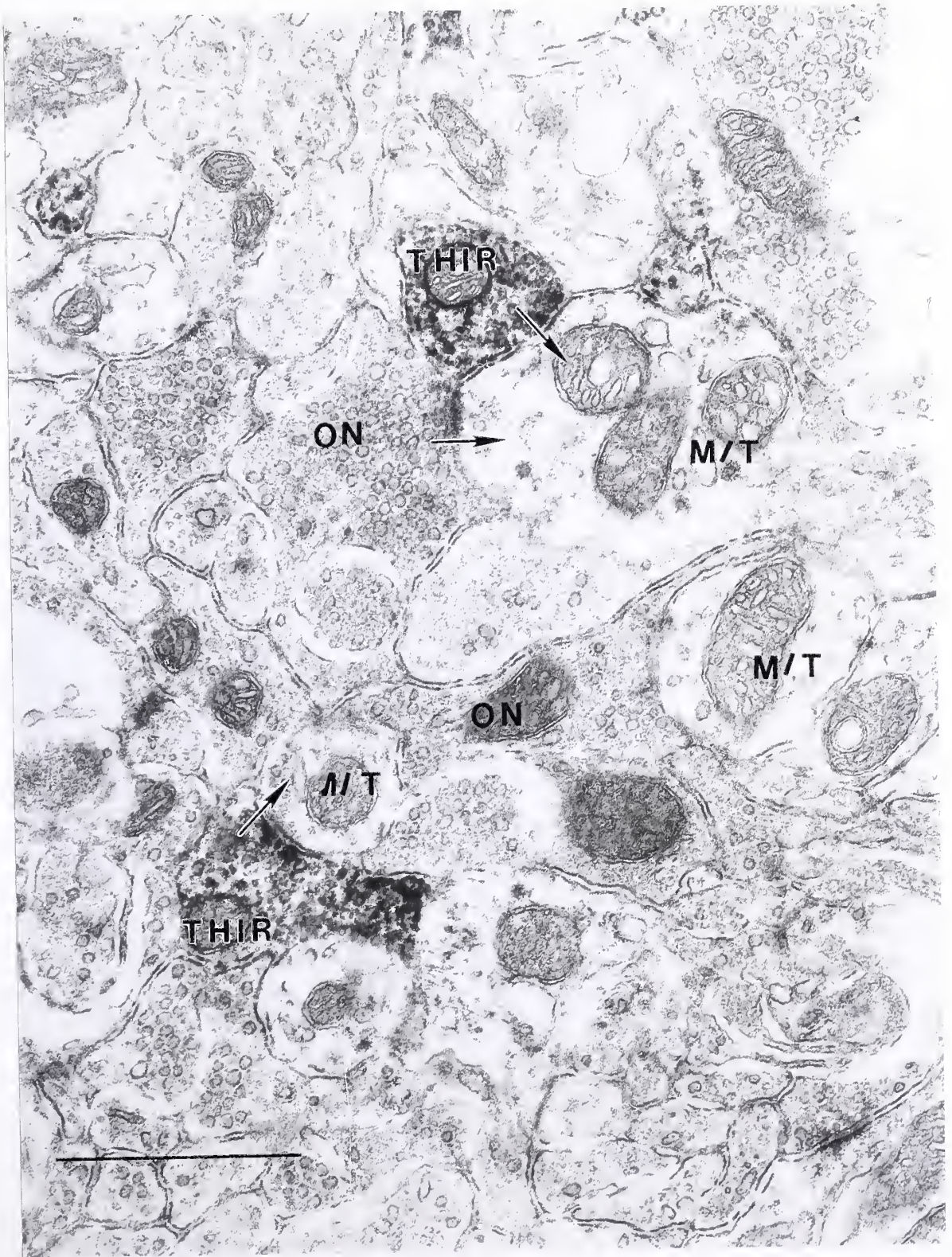








Figure 42. Electron micrograph of rat olfactory bulb immunohistochemically stained using anti-tyrosine hydroxylase primary antibodies. Another illustration of a tyrosine hydroxylase immunoreactive process making type II, symmetrical synapse onto a longitudinally cut mitral/tufted cell dendritic process. This synapse is made in the vicinity of a larger immunoreactive process. Arrows indicate direction or polarity of synapse. Abbreviations M/T, mitral/tufted dendritic process; THIR, tyrosine hydroxylase immunoreactive process. Scale bar= 1 $\mu$ m.

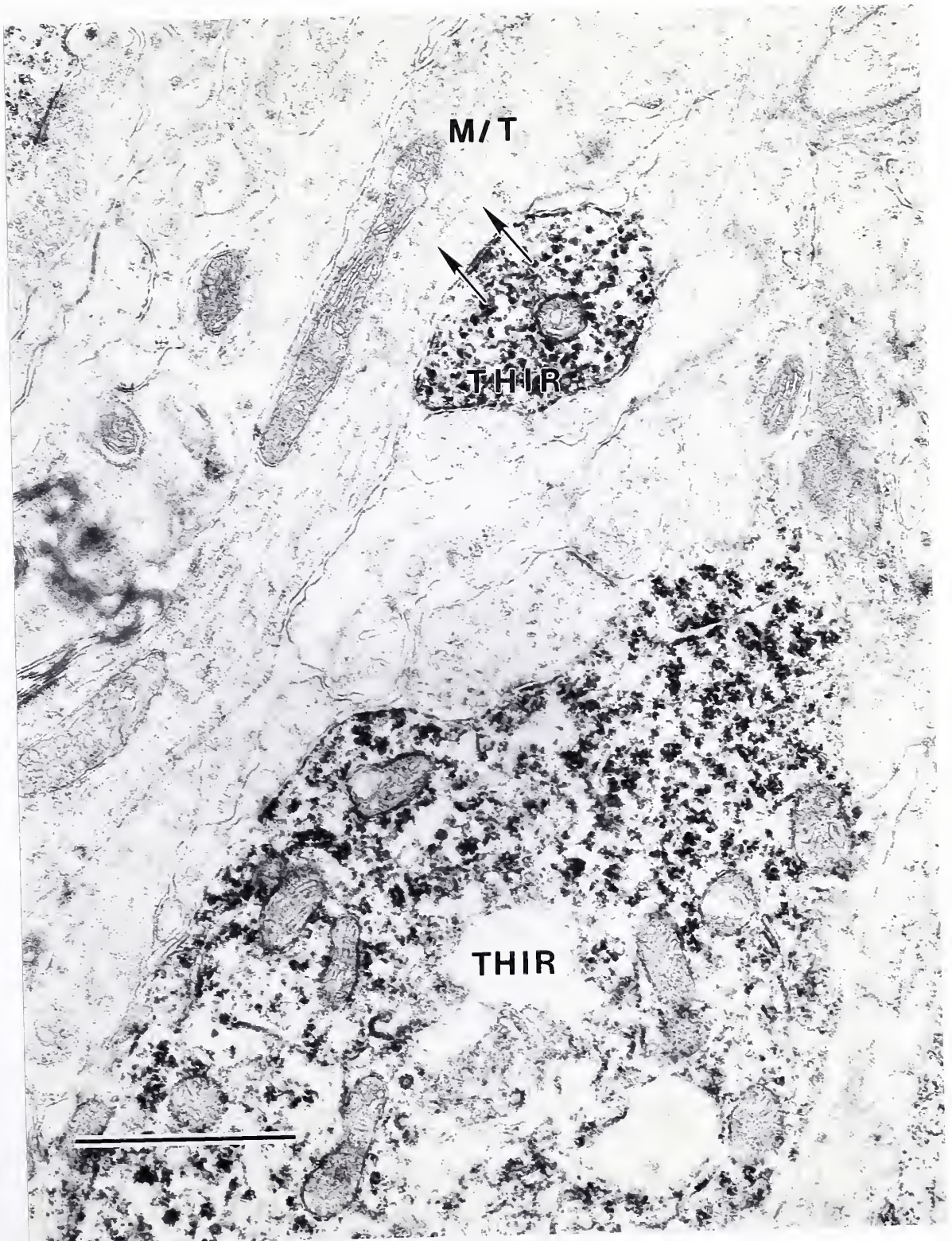
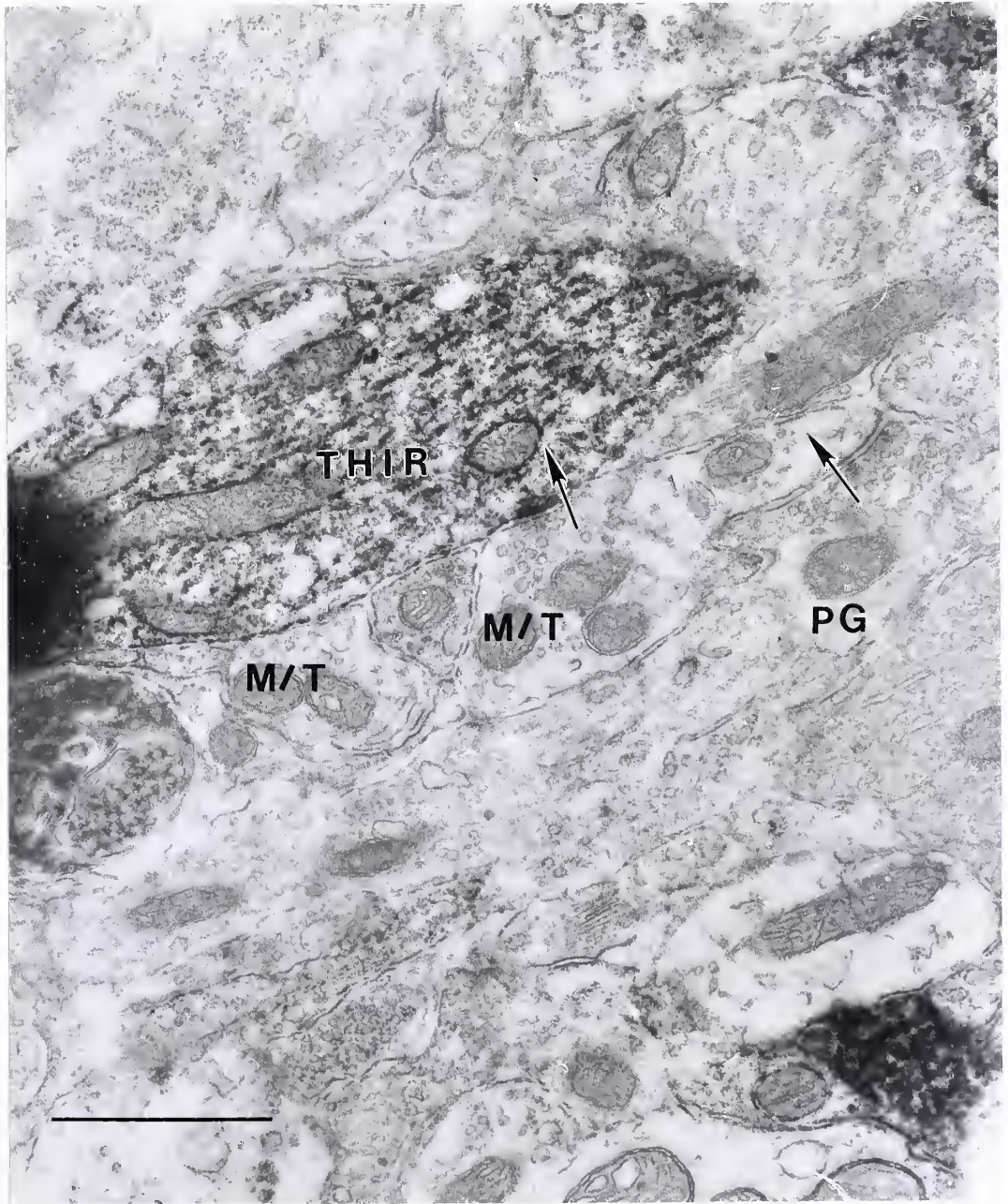






Figure 43. Electron micrograph of rat olfactory bulb immunohistochemically stained using anti-tyrosine hydroxylase primary antibodies. A periglomerular cell dendritic process makes a type II, symmetrical synapse in the vicinity of an immunoreactive process onto a mitral/tufted cell dendritic process. In turn this mitral/tufted cell process establishes a type I, asymmetrical synapse onto the immunoreactive process. This collectively forms a circuit where the periglomerular cell process inhibits the release of the mitral/tufted cell vesicles that would consequentially excited the immunoreactive process. Arrows indicate direction or polarity of synapse. Abbreviations: M/T, mitral/tufted dendritic process; THIR, tyrosine hydroxylase immunoreactive process; PG, periglomerular cell dendritic process. Scale bar= 1 $\mu$ m.









process making a type I synapse suggestive of a tyrosine hydroxylase immunoreactive superficial tufted cell.

It was very difficult to find reciprocal synapses involving mitral/tufted and periglomerular cell dendritic processes. It became more difficult to identify these synapses when a dark profile such as the immunoreactive ones were involved. In figure 44 an olfactory nerve terminal makes an asymmetrical synapse onto a mitral/tufted cell dendrite. The mitral/tufted cell dendrite is involved in a reciprocal synapse with an immunoreactive periglomerular cell. Therefore the processing of information goes from the olfactory nerve (excitation)→ mitral/tufted cell dendrite (excitation)→ periglomerular cell dendrite (inhibition)→ mitral/tufted cell dendrite. Reciprocal synapses involving a non-immunoreactive process was also identified (Figure 45). Notice the large mitral/tufted cell dendrite making an asymmetrical synapse onto a non-immunoreactive periglomerular cell dendrite. The periglomerular cell dendrite in turn makes a symmetrical synapse onto the mitral/tufted cell dendrite.

## 2. GABA

GABA immunoreactive cell bodies has a similar staining pattern as that observed in tyrosine hydroxylase immunoreactive cell bodies. However, the peroxidase immunoreaction for GABA did not yield as strong reaction as seen for tyrosine hydroxylase. Using low magnification photographs of areas under study, we were able to assess GABAergic immunoreactive cell bodies, when equivocal stained cell bodies were visualized under the electron microscope. For this reason GABA immunoreactive cell bodies are not illustrated in any figures. Sufficient to say is that morphologically under the light and electron microscope these immunoreactive cell bodies were identified as periglomerular cells.

Olfactory nerve terminals made type I, asymmetrical synapses onto a mitral/tufted cell dendritic process in the vicinity of GABA immunoreactive processes (GIR, in figure





Figure 44. Electron micrograph of rat olfactory bulb immunohistochemically stained using anti-tyrosine hydroxylase primary antibodies. Illustration of a synaptic triad involving an olfactory nerve terminal making a type I, asymmetrical synapse onto a mitral/tufted cell dendritic process. The mitral/tufted cell process is engaged in a reciprocal synapse with the tyrosine hydroxylase immunoreactive process. Arrows indicate direction or polarity of synapse. Abbreviations: ON, olfactory nerve terminal; M/T, mitral/tufted dendritic process; THIR, tyrosine hydroxylase immunoreactive process. Scale bar= 1 $\mu$ m.











Figure 45. Electron micrograph of rat olfactory bulb immunohistochemically stained using anti-tyrosine hydroxylase primary antibodies. Non-immunoreactive periglomerular cell dendritic processes were also involved in establishing reciprocal synapse. A mitral/tufted cell dendritic process makes a type I synapse onto the periglomerular cell dendritic processes. In turn the periglomerular cell dendritic processes makes a type II synapse back onto the mitral/tufted cell process. Notice that this synapse is in the vicinity of an immunoreactive process. Arrows indicate direction or polarity of synapse. Abbreviations: ON, olfactory nerve terminal; M/T, mitral/tufted dendritic process; THIR, tyrosine hydroxylase immunoreactive process; PG, periglomerular cell dendritic process. Scale bar= 1 $\mu$ m.

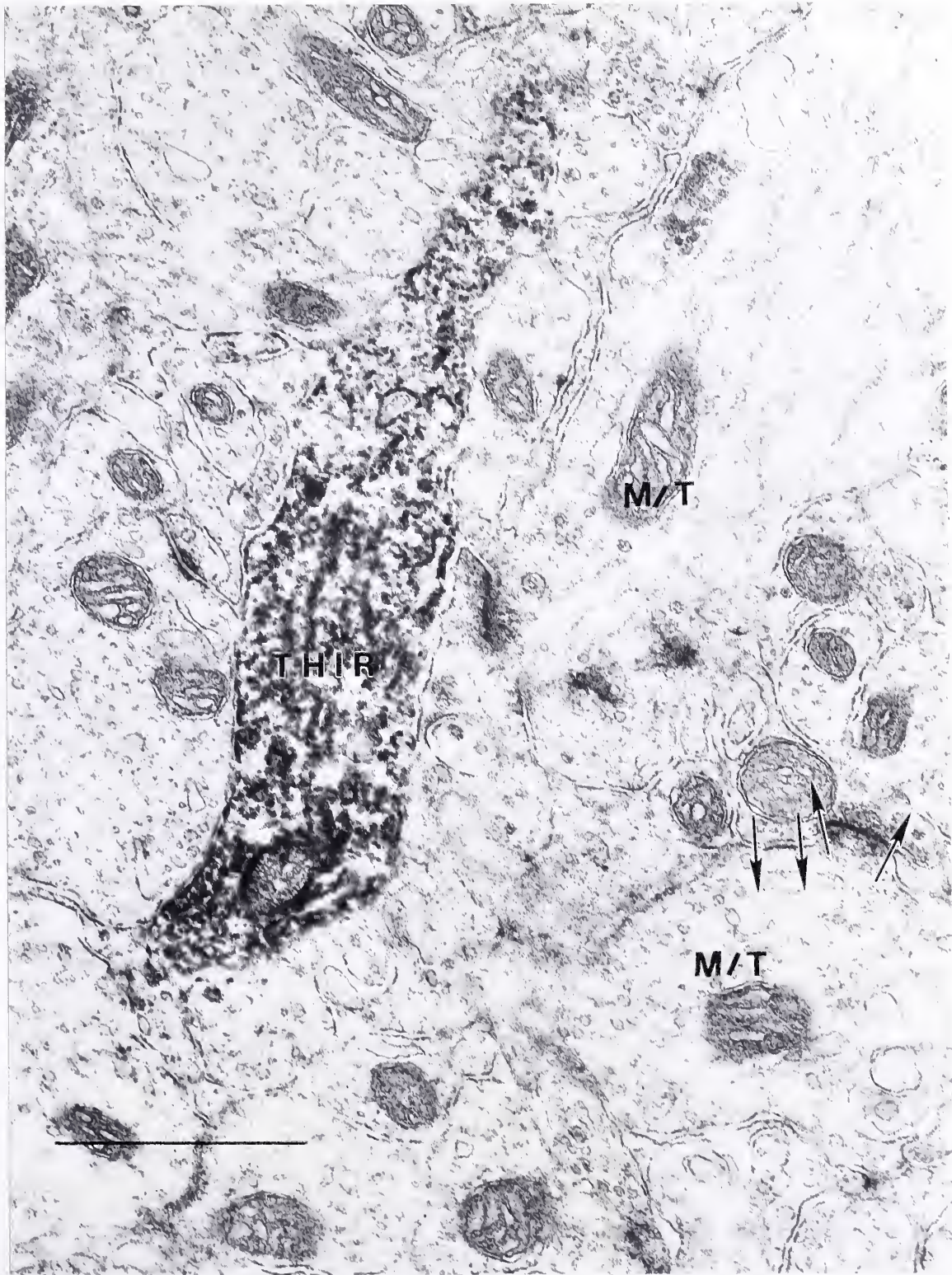






Figure 46. Electron micrograph of rat olfactory bulb immunohistochemically stained using anti-GABA primary antibodies. Olfactory nerve terminal establishes a type I, asymmetrical synapse onto a pale non-immunoreactive dendritic presumably a mitral/tufted cell process. Notice morphological characteristics of olfactory nerve terminal with electron dense and vesicle filled axoplasm. Arrows indicate direction or polarity of synapse. Abbreviations: ON, olfactory nerve terminal; M/T, mitral/tufted dendritic process; GIR, GABA immunoreactive process. Scale bar= 0.5 $\mu$ m.



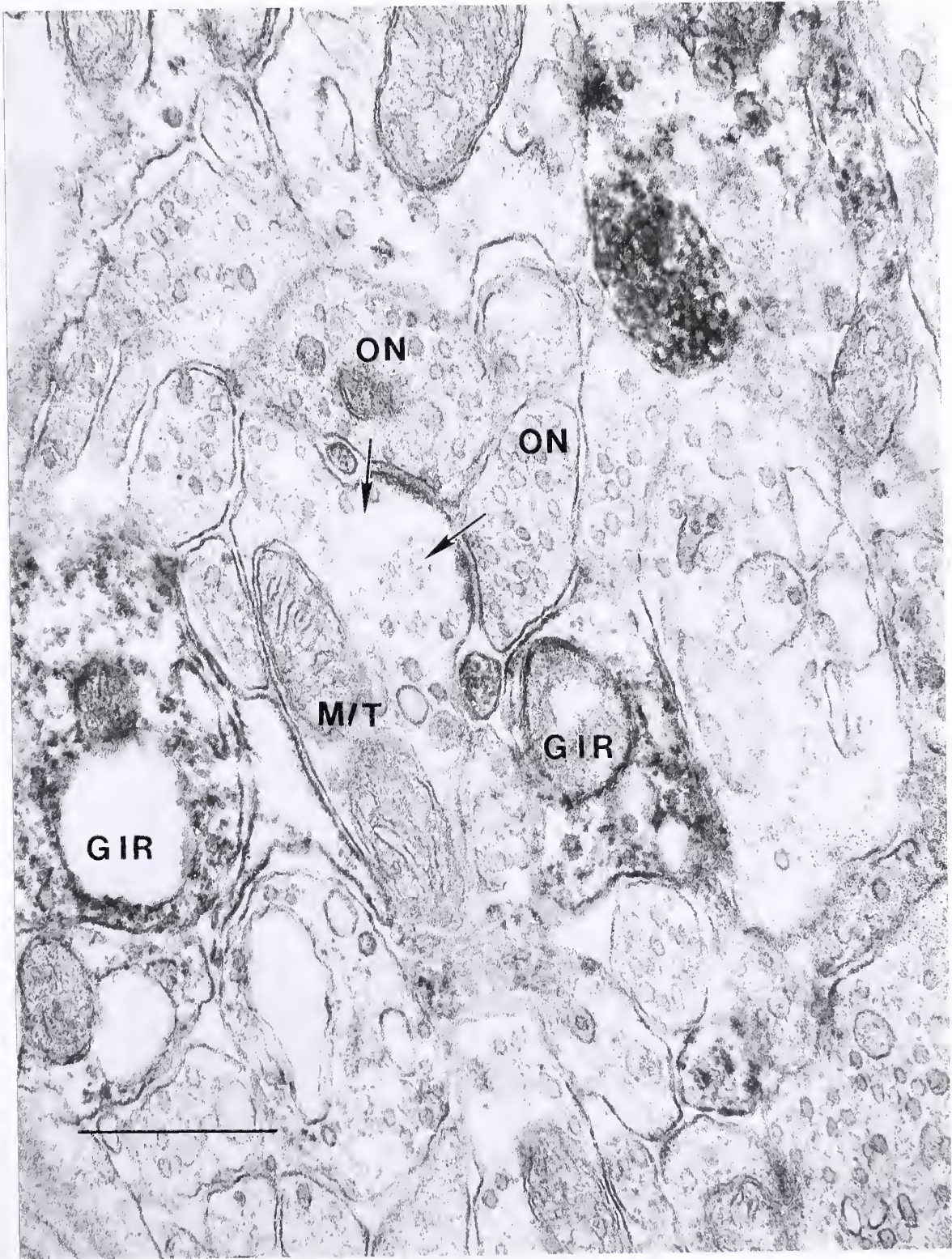
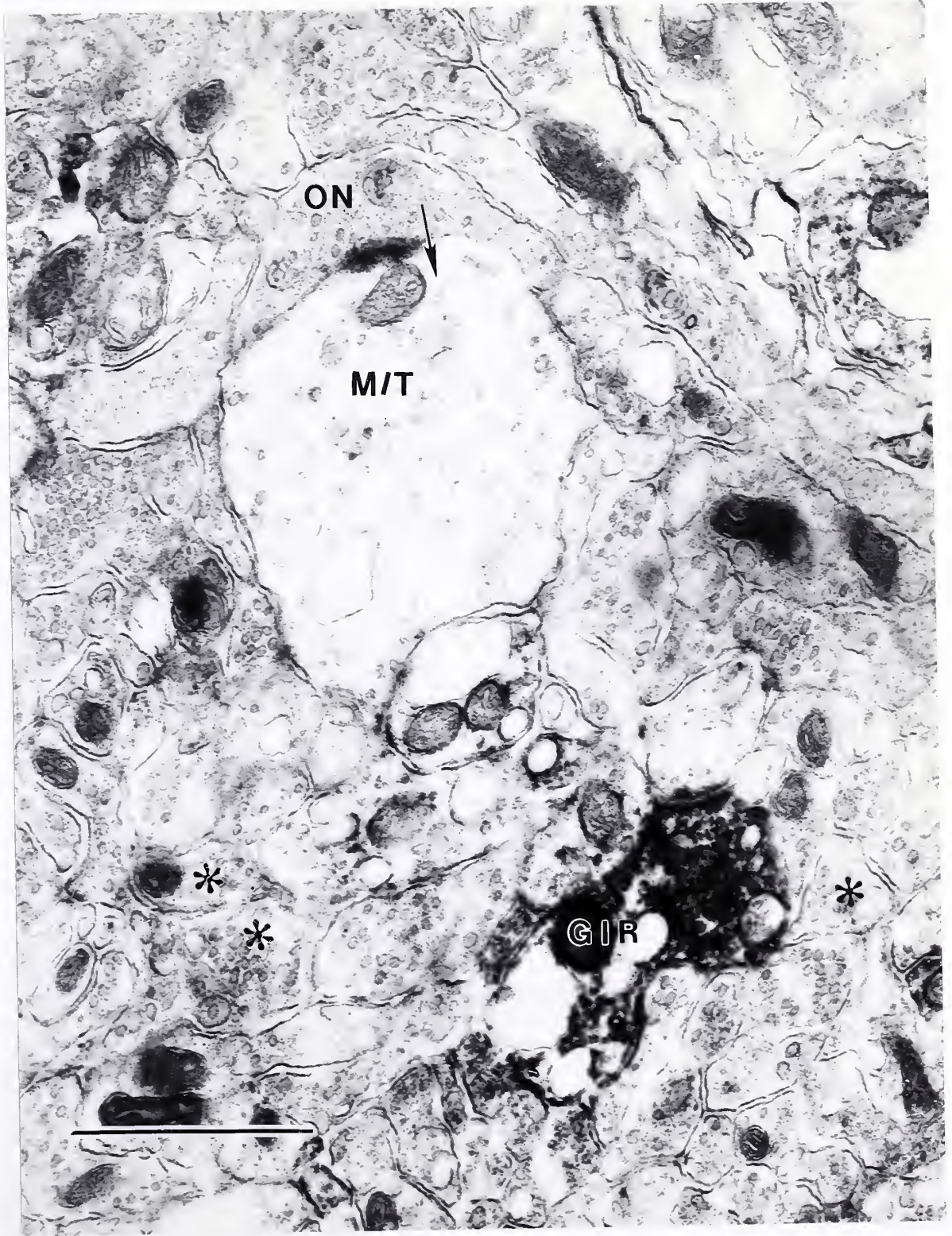








Figure 47. Electron micrograph of rat olfactory bulb immunohistochemically stained using anti-GABA primary antibodies. Illustrating another example of an olfactory nerve terminal establishing a type I, asymmetrical synapse onto a pale non-immunoreactive dendritic presumably a mitral/tufted cell process. Arrows indicate direction or polarity of synapse, asterisk indicate cross sectionally cut olfactory nerve axon. Abbreviations: ON, olfactory nerve terminal; M/T, mitral/tufted dendritic process; GIR, GABA immunoreactive process. Scale bar= 1 $\mu$ m.







46 and 47). Multiple olfactory nerve terminals would converge more frequently onto mitral/tufted cell processes as compared to immunoreactive and non-immunoreactive olfactory nerve terminals or from dendritic processes onto olfactory nerve terminals . Figure 48 illustrates olfactory nerve terminals making type I synapses onto GABA immunoreactive and non-immunoreactive periglomerular cell dendritic processes. Periglomerular cell dendritic processes exhibited a darker cytoplasm, compared to mitral/tufted dendritic processes. These dendritic processes often had several intracytoplasmic organelles and contained numerous pleomorphic vesicles (arrow head in Figure 48) interspersed within the cytoplasm. The GABA immunoreactive processes also contained intracytoplasmic organelles and also evident is the presence of pleomorphic vesicles interspersed within the dendritic process.

GABA immunoreactive processes were also involved in dendrodendritic synapses with mitral/tufted cell dendritic processes as shown in figures 49 and 50. In figure 49 the mitral/tufted cell dendrite is simultaneously receiving a type I synapse from an olfactory nerve terminal. Figure 50 shows large mitral/tufted cell dendritic process making several type I synapses onto a GABA immunoreactive process and periglomerular cell dendritic processes. Notice also that a periglomerular cell dendritic process receives an asymmetrical synapse from a mitral/tufted cell dendrite.

GABA immunoreactive processes established conventional type II, symmetrical synapses. In figure 51 two GABA immunoreactive processes make symmetrical synapse onto a longitudinally cut mitral/tufted cell dendrite and the other onto a smaller, darker axon like postsynaptic target (A in Figure 51). It should be emphasized at this juncture that not all postsynaptic targets were clearly identified. These targets were labeled as unidentified and were separated under another category for the quantitative analysis (see below and under Table 2). Another conventional type II synapse is shown in figure 52, where a GABA immunoreactive process makes a symmetrical synapse onto a







Figure 48. Electron micrograph of rat olfactory bulb immunohistochemically stained using anti-GABA primary antibodies. Olfactory nerve terminal establishes type I, asymmetrical synapses onto a GABA immunoreactive dendritic process and onto a non-immunoreactive presumed periglomerular cell dendritic process. Notice similarities between immunoreactive and non-immunoreactive periglomerular cell processes such as numerous pleomorphic vesicles interspersed within cytoplasm (arrow heads). Arrows indicate direction or polarity of synapse, asterisk indicate cross sectionally cut olfactory nerve axon. Abbreviations: ON, olfactory nerve terminal; M/T, mitral/tufted dendritic process; GIR, GABA immunoreactive process; PG, periglomerular cell dendritic process. Scale bar= 1 $\mu$ m.

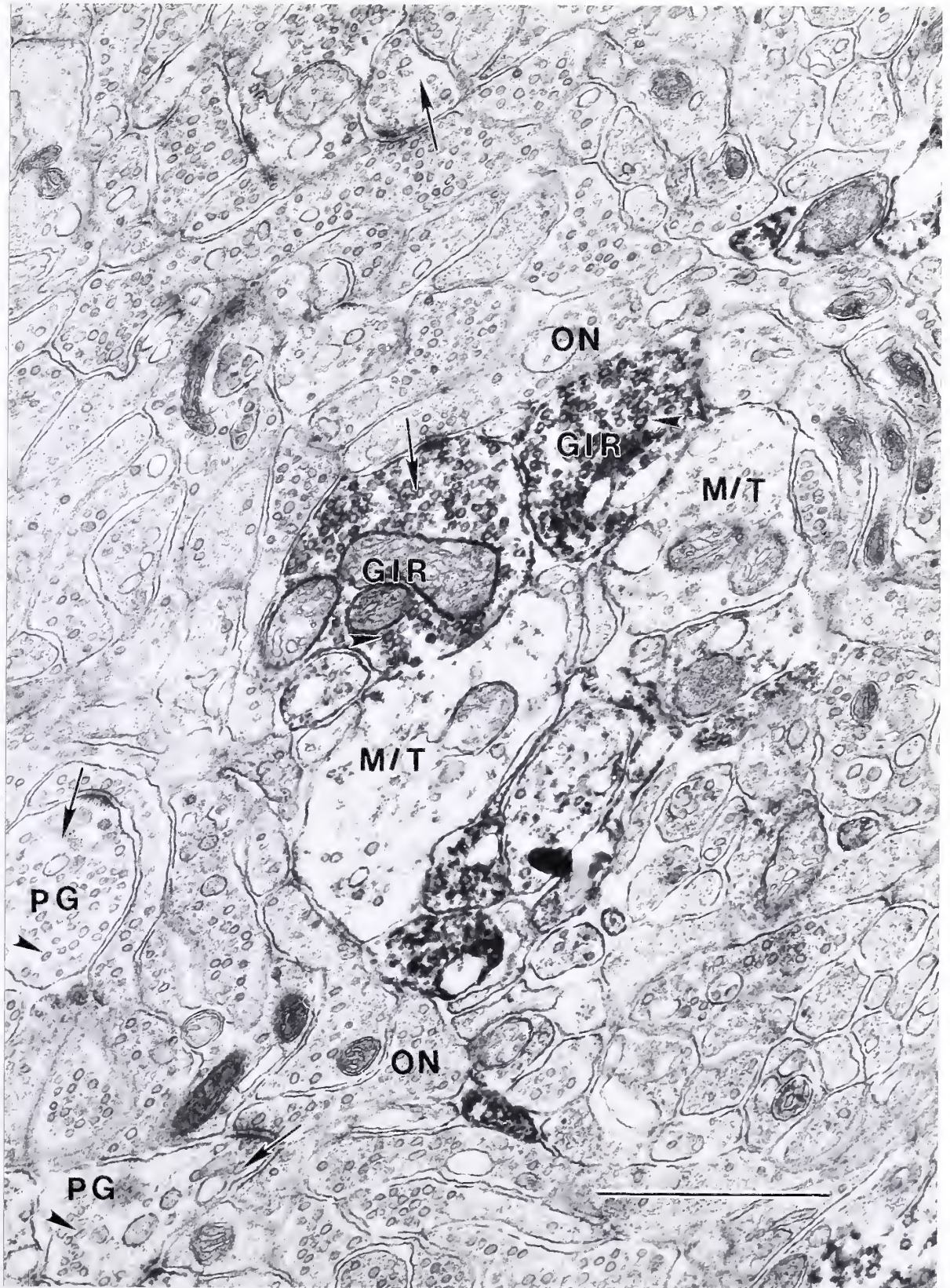








Figure 49. Electron micrograph of rat olfactory bulb immunohistochemically stained using anti-GABA primary antibodies. Dendrodendritic synapse between immunoreactive and a mitral/tufted cell dendritic processes. Mitral/tufted cell dendritic process make type I asymmetrical synapses onto immunoreactive and non-immunoreactive periglomerular cell dendritic processes. Also shown is an olfactory nerve terminal making an asymmetrical synapses onto the mitral/tufted cell dendritic process. Arrows indicate direction or polarity of synapse. Abbreviations: ON, olfactory nerve terminal; M/T, mitral/tufted dendritic process; GIR, GABA immunoreactive process. Scale bar= 1 $\mu$ m.

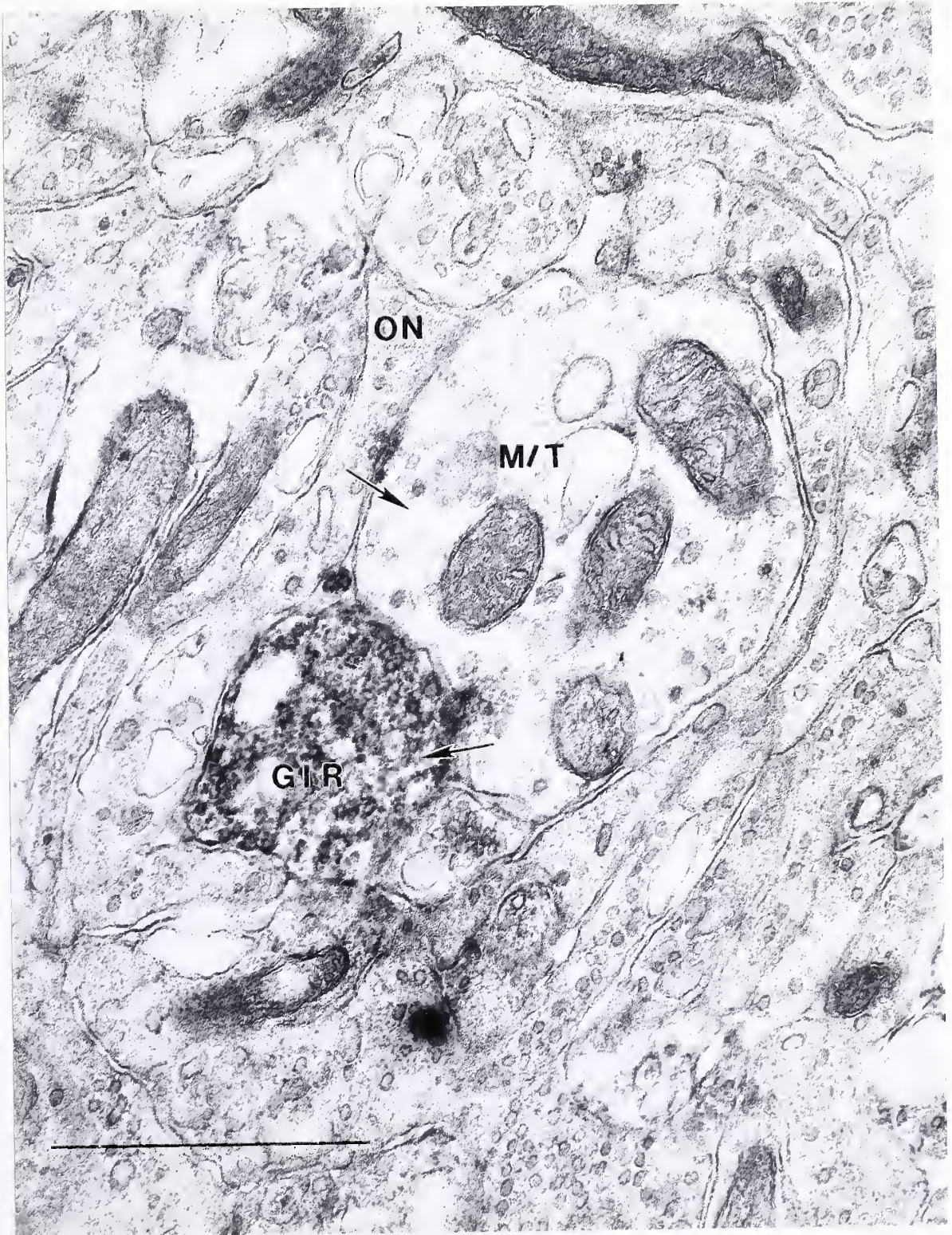








Figure 50. Electron micrograph of rat olfactory bulb immunohistochemically stained using anti-GABA primary antibodies. Dendrodendritic synapse between immunoreactive and a mitral/tufted cell dendritic processes. Another large mitral/tufted cell process makes a type I, asymmetrical synapses onto a presumed periglomerular cell dendritic process (asterisk). . Arrows indicate direction or polarity of synapse, asterisk indicates a periglomerular cell dendritic process. Abbreviations: M/T, mitral/tufted dendritic process; GIR, GABA immunoreactive process. Scale bar= 1 $\mu$ m.

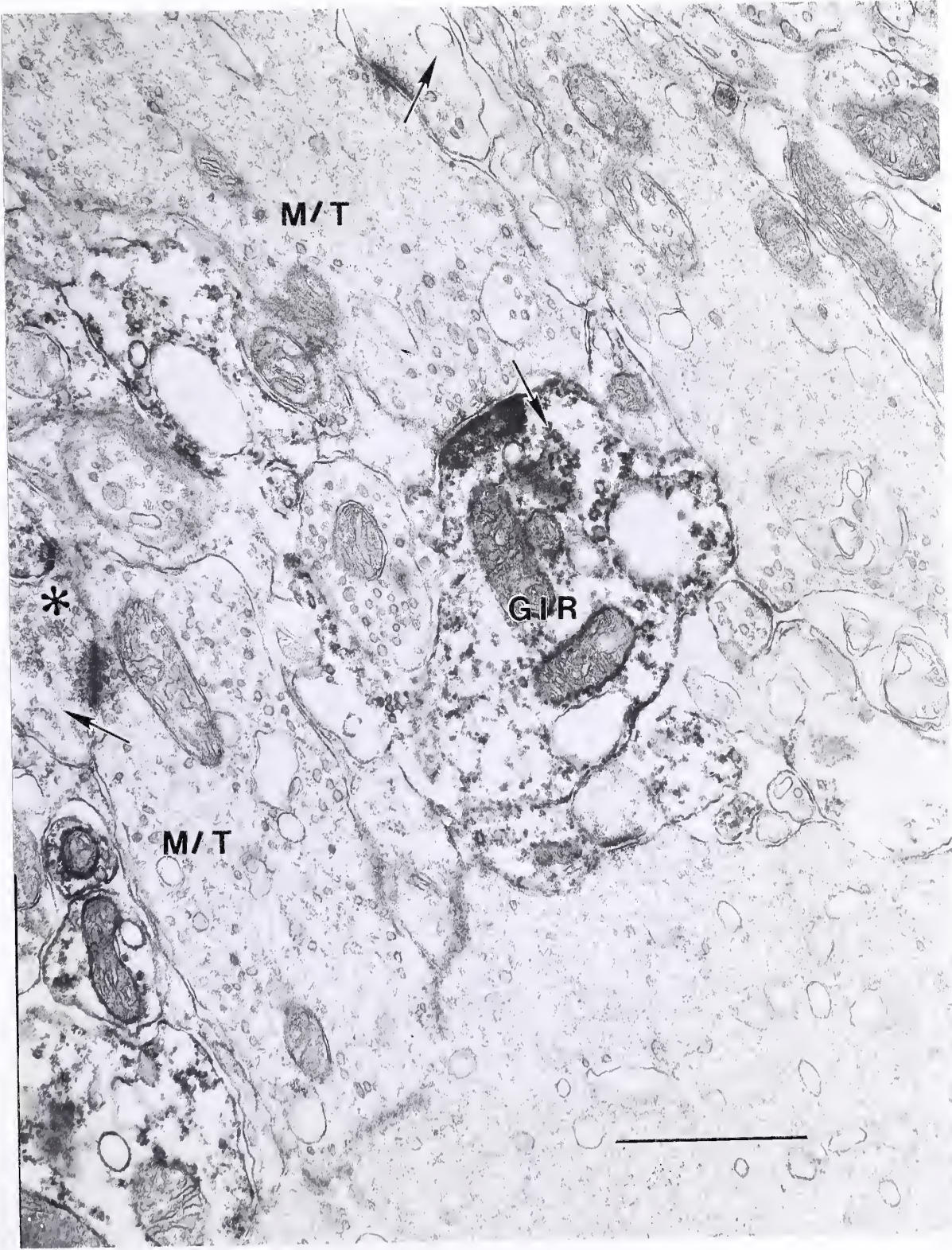






Figure 51. Electron micrograph of rat olfactory bulb immunohistochemically stained using anti-GABA primary antibodies. GABA immunoreactive dendritic processes make type II symmetrical synapses. The lower immunoreactive process makes a symmetrical synapses onto a longitudinally cut mitral/tufted cell dendrite. The other immunoreactive process makes a symmetrical synapses onto an unidentified axon-like postsynaptic target. Arrows indicate direction or polarity of synapse. Abbreviations: M/T, mitral/tufted dendritic process; GIR, GABA immunoreactive process; A, axon. Scale bar= 1 $\mu$ m.



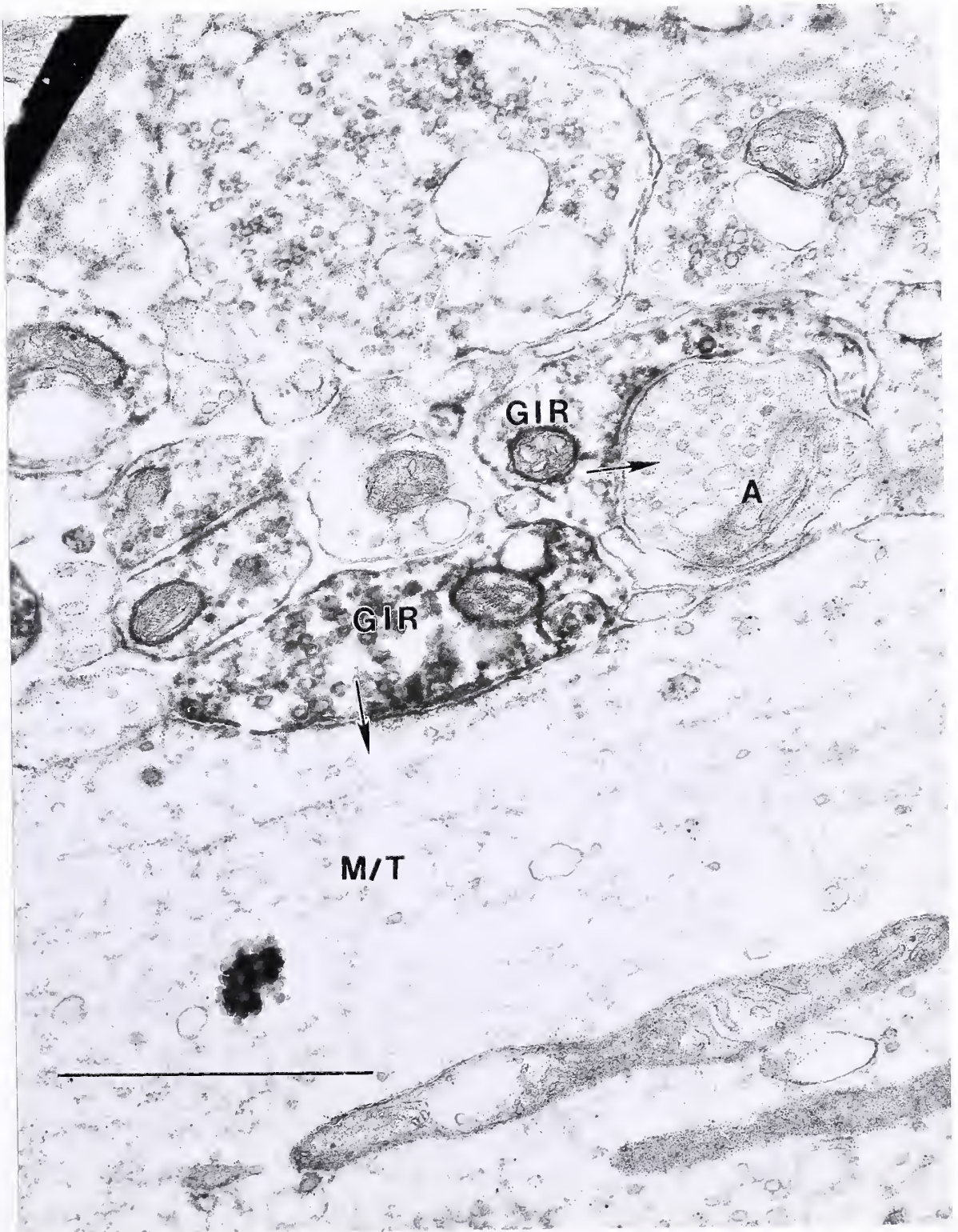










Figure 52. Electron micrograph of rat olfactory bulb immunohistochemically stained using anti-GABA primary antibodies. GABA immunoreactive dendritic process makes type II, symmetrical synapse onto a mitral/tufted cell dendritic process. Also shown in top of micrograph a mitral/tufted cell dendrite makes a type I synapse onto a immunoreactive process. In lower part of micrograph another example but this time the mitral/tufted cell dendrite makes a synapse onto a non-immunoreactive periglomerular cell. Arrows indicate direction or polarity of synapse. Abbreviations: M/T, mitral/tufted dendritic process; GIR, GABA immunoreactive process; Asterisk, indicates periglomerular cell dendritic process. Scale bar= 1 $\mu$ m.

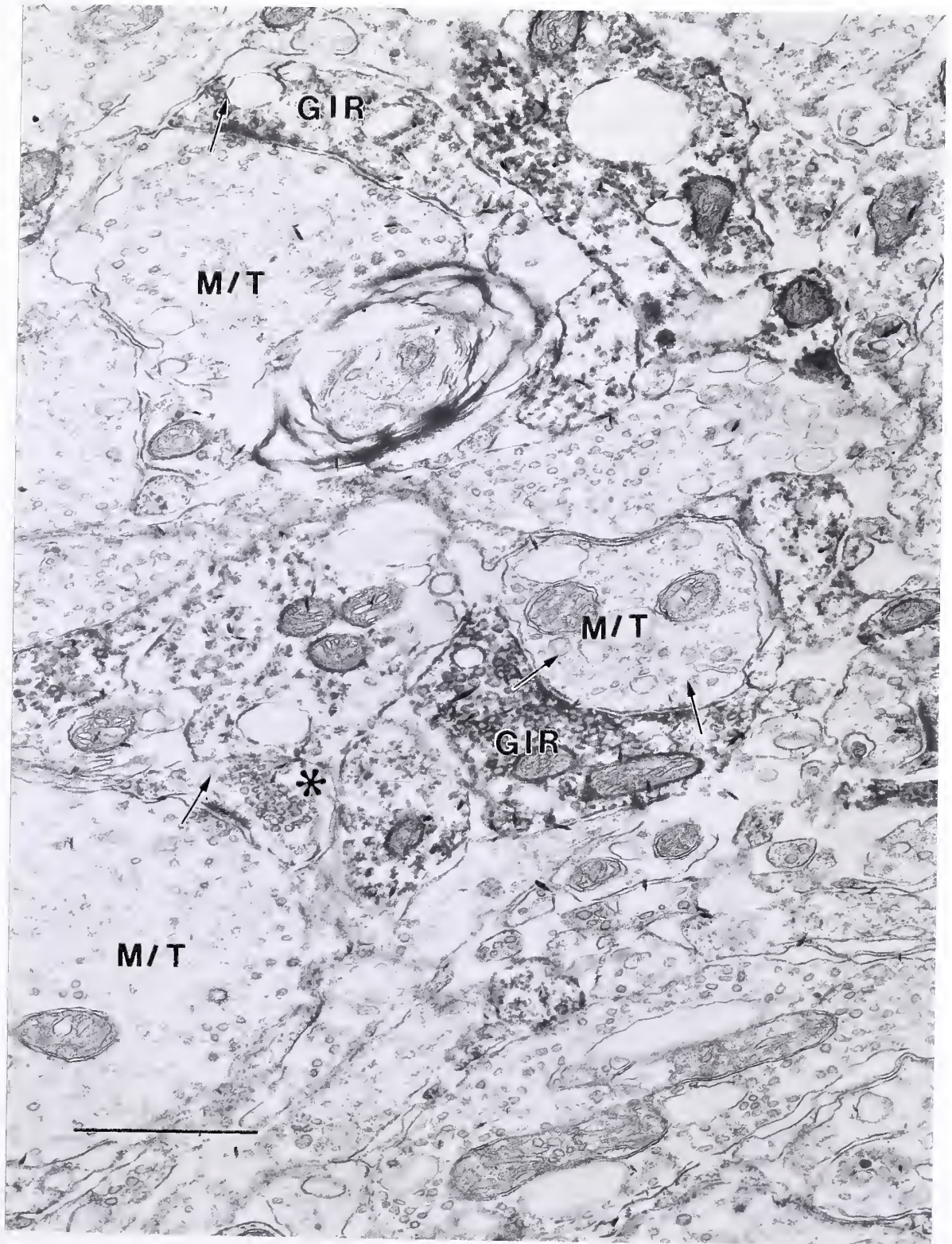
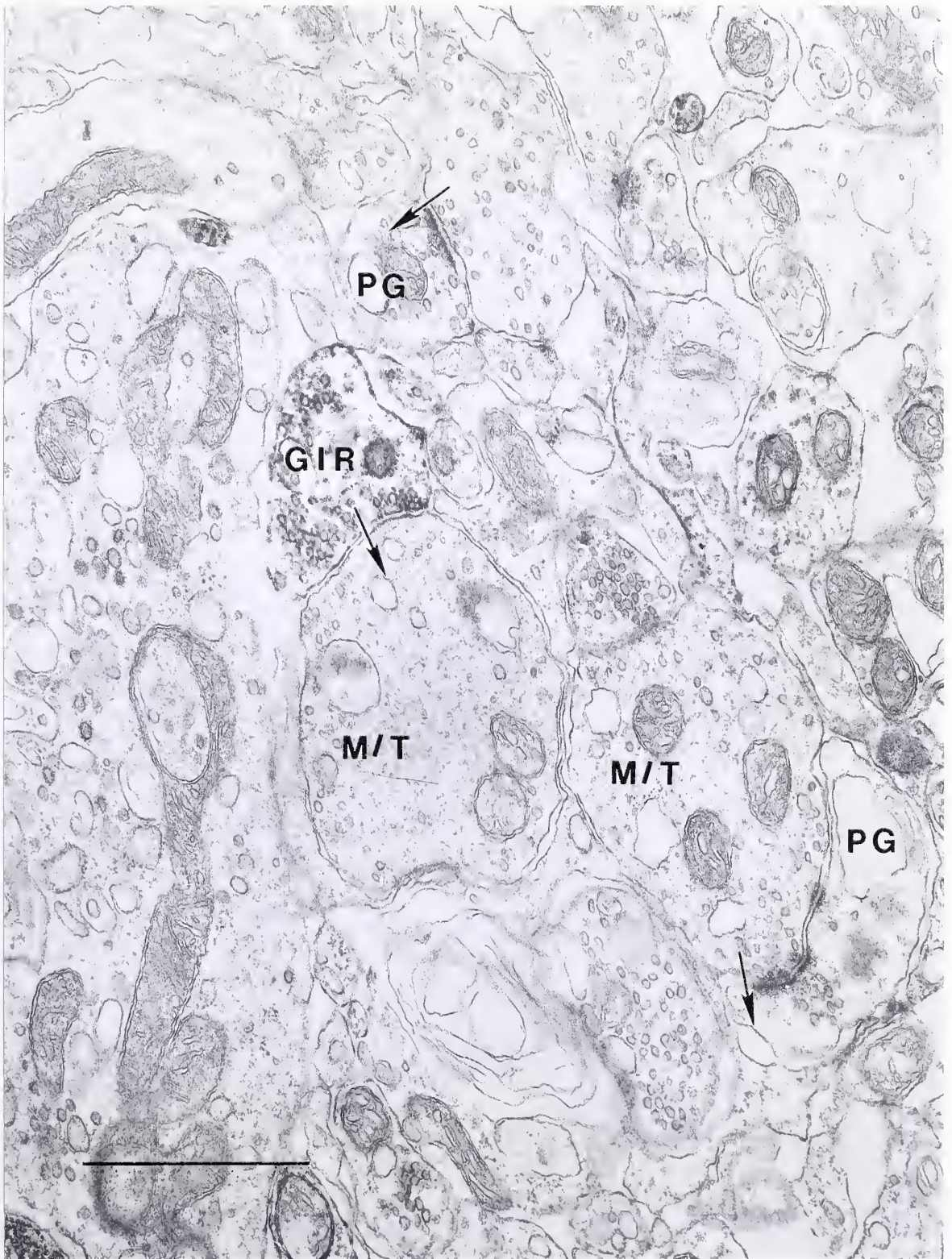








Figure 53. Electron micrograph of rat olfactory bulb immunohistochemically stained using anti-GABA primary antibodies. Illustration of a GABA immunoreactive dendritic process making type II, symmetrical synapse onto a mitral/tufted cell dendritic process. Also shown in micrograph are two examples of mitral/tufted cell dendrites making a type I synapse onto a none-immunoreactive periglomerular cell processes. Arrows indicate direction or polarity of synapse. Abbreviations: M/T, mitral/tufted dendritic process; GIR, GABA immunoreactive process; PG, periglomerular cell dendritic process. Scale bar= 1 $\mu$ m.





mitral/tufted cell dendritic process (M/T). Figure 53 illustrate two examples of symmetrical, type II synapses from a GABA immunoreactive process and a non-immunoreactive periglomerular cell process. A closer look at the GABA immunoreactive process reveals the presence of two synaptic vesicles opening into the synaptic cleft. Better illustrations of type II synapses from non-immunoreactive periglomerular cell processes are presented in figures 54 and 55. Notice the collection of pleomorphic vesicles in the presynaptic periglomerular cell dendritic process juxtaposed to a symmetrical postsynaptic membrane specialization. The presence of this synapse in the vicinity of a GABA immunoreactive process supports the notion that both immunoreactive and non-immunoreactive processes are involved in making type II synapses. This is further exemplified in figure 55 where a periglomerular cell dendritic process makes a symmetrical synapse onto a mitral/tufted cell dendrite . There was no evidence of synaptic interaction between two periglomerular cell dendritic processes. Likewise there was no synaptic interaction between two GABA immunoreactive processes or between a GABA immunoreactive and a periglomerular cell dendritic process.

### 3. Quantitative

A total of 1,209 dendritic processes were identified in the tyrosine hydroxylase study and 1,059 dendritic processes in the GABA study. Of these processes, 788 and 338, respectively either received or made synapses. Table 2 shows a summary of these processes and their distribution according to their type and pre-and postsynaptic morphological profiles .

Of the 788 processes in the tyrosine hydroxylase study receiving or making synapses, 433 involved mitral/tufted cell processes. From the mitral/tufted cell group 185 (42.2%) received a type I, asymmetrical synapse from olfactory nerve axons (AXODEN in Table 2); 102 (23.4%) made type I asymmetrical synapses onto non-immunoreactive









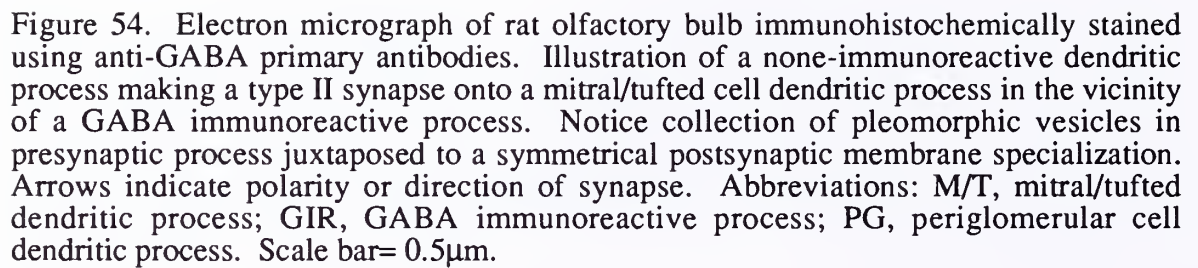


Figure 54. Electron micrograph of rat olfactory bulb immunohistochemically stained using anti-GABA primary antibodies. Illustration of a none-immunoreactive dendritic process making a type II synapse onto a mitral/tufted cell dendritic process in the vicinity of a GABA immunoreactive process. Notice collection of pleomorphic vesicles in presynaptic process juxtaposed to a symmetrical postsynaptic membrane specialization. Arrows indicate polarity or direction of synapse. Abbreviations: M/T, mitral/tufted dendritic process; GIR, GABA immunoreactive process; PG, periglomerular cell dendritic process. Scale bar= 0.5µm.

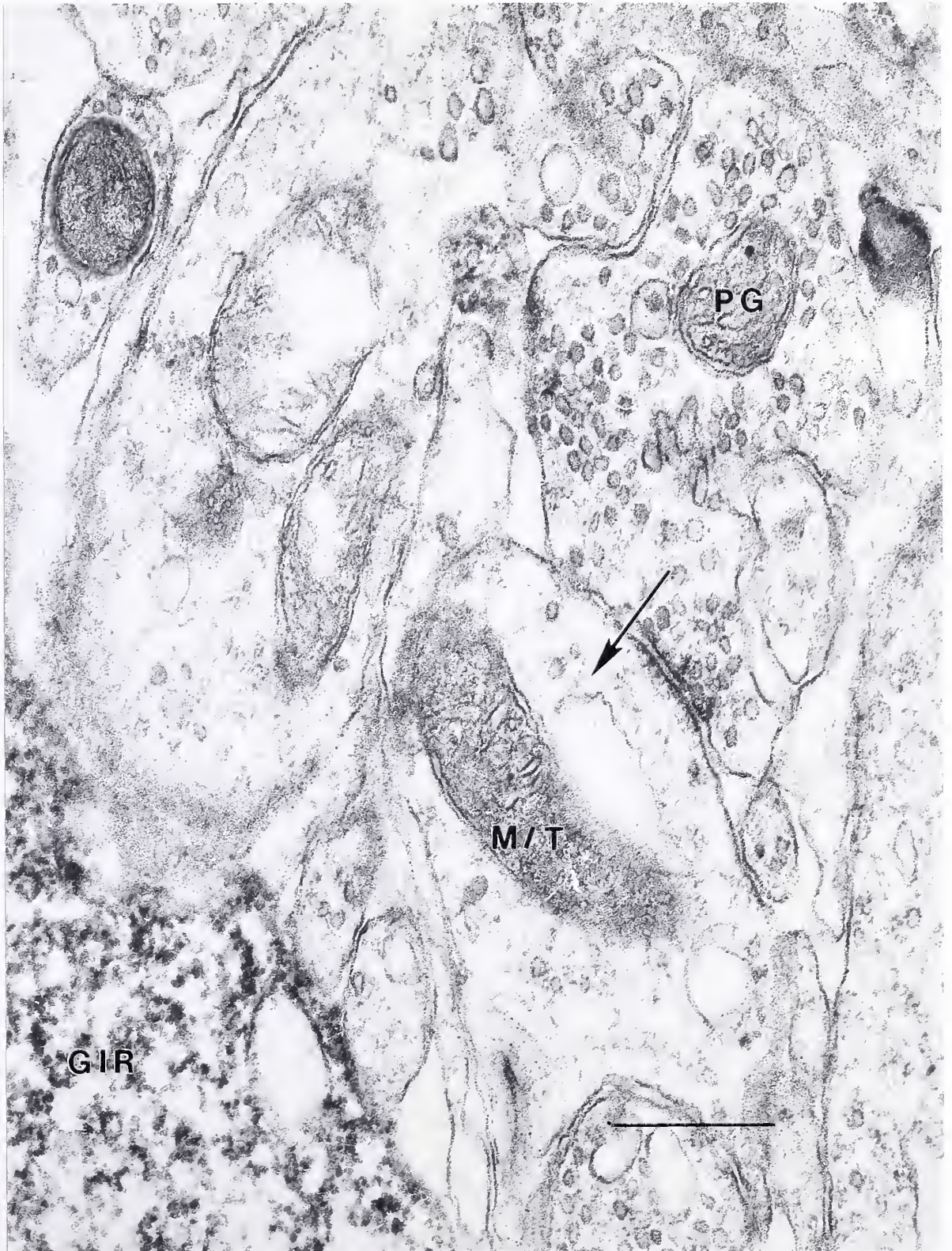
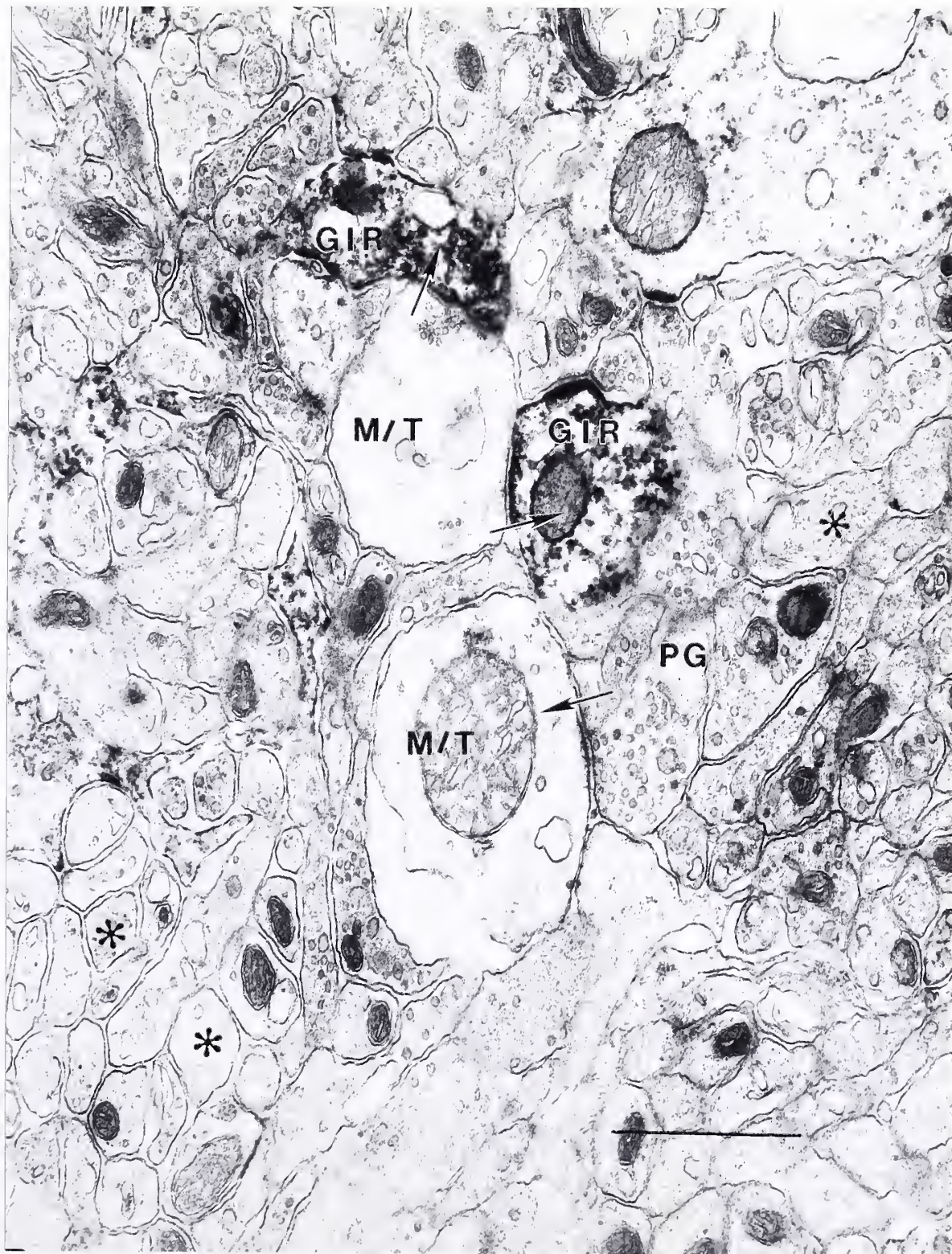






Figure 55 Electron micrograph of rat olfactory bulb immunohistochemically stained using anti-GABA primary antibodies. Another example of a none-immunoreactive dendritic process making a type II, synapse onto a mitral/tufted cell dendritic process in the vicinity of several GABA immunoreactive processes. Also shown is a mitral/tufted cell dendritic process making two type I synapses onto two different GABA immunoreactive processes. Arrows indicate polarity or direction of synapse, and asterisk indicate cross sectionally cut olfactory nerve axons. Abbreviations: M/T, mitral/tufted dendritic process; GIR, GABA immunoreactive process; PG, periglomerular cell dendritic process. Scale bar= 1 $\mu$ m.









		TOTAL	AXODEN	DENDRODENDRITIC ASYM                  SYM		?
TH	M/T	433	185 (42.1%)	102 (23.4%)	145 (33.3%)	6 (1.2%)
	PG-NIR	162	47 (29%)	45 (27.7%)	67 (41.3%)	3 (1.8%)
	TH-IR	193	58 (30%)	73 (37.8%)	62 (32.1%)	1 (0.5%)
GABA	M/T	172	57 (33.1%)	21 (12.2%)	93 (54.0%)	1 (0.5%)
	PG-NIR	79	13 (16.4%)	50 (63.2%)	16 (20.2%)	
	GABA-IR	87	19 (21.8%)	53 (60.9%)	15 (17.2%)	

Table 2. Synaptological analysis of immunoreactive, non-immunoreactive, and mitral/tufted cell dendritic processes. AXON refers to axodendritic synapses from olfactory nerve terminals onto dendritic processes. DENDRODENDRITIC, refers to synapses made between immunoreactive, non-immunoreactive, and mitral/tufted cell dendritic processes. ASYM refers to asymmetrical, type I synapses and SYM refers to symmetrical, type II synapses. Abbreviations: GABA-IR, GABA immunoreactive processes; TH-IR, tyrosine hydroxylase, immunoreactive processes; PG-NIR, non-immunoreactive periglomerular cell processes; M/T, mitral/tufted cell dendritic processes.



and immunoreactive periglomerular cell processes; 145 (33.3%) received type II, symmetrical synapses from immunoreactive and non-immunoreactive periglomerular cell processes. Of the 162 non-immunoreactive (PG N-IR in Table 2) involved in receiving or making synapses; 47 (29%) received type I, asymmetrical synapses from olfactory nerve terminals; 45 (27.7%) received type I, asymmetrical synapses from mitral/tufted cell processes; 67 (41.3%) made type II, symmetrical synapses onto mitral/tufted cell dendritic processes. Of the 193 tyrosine hydroxylase immunoreactive processes (TH-IR in Table 2) involved in receiving or making synapses; 58 (30%) received a type I, asymmetrical synapse from olfactory nerve terminals; 73 (37.8%) received asymmetrical synapses from mitral/tufted cell dendritic processes; and 62 (32.1%) made symmetrical synapse onto mitral/tufted cell dendritic profiles.

Of the 343 processes in the GABA study receiving or making synapses, 172 involved mitral/tufted cell processes. From the mitral/tufted cell group, 57 (33.1%) received type I, asymmetrical synapses from the olfactory nerve terminals; 93 (54%) made type I, asymmetrical synapses onto non-immunoreactive and immunoreactive periglomerular cell processes; 21 (12.2%) received type II, symmetrical synapses from non-immunoreactive and immunoreactive periglomerular cell processes. Of the 79 non-immunoreactive periglomerular cell processes involved in receiving or making synapses; 13 (16.4%) received type I, asymmetrical synapses from olfactory nerve terminals; 50 (63.2%) received asymmetrical synapses from mitral/tufted cell processes; and 16 (20%) established symmetrical synapses onto mitral/tufted cell profiles. Finally there were 87 GABA immunoreactive processes involved in making or receiving synapses; 19 (21.8%) received asymmetrical synapses from olfactory nerve terminals; 53 (60.9%) received asymmetrical synapses from mitral/tufted cell profiles; and 15 (17.2%) made symmetrical synapses onto mitral/tufted cell profiles. As shown in Table 2 there were several synapses involving unidentifiable profiles.



## G. SUMMARY

The aim of this study was to characterize the synaptic organization of periglomerular cells processes immunoreactive for tyrosine hydroxylase and GABA. Morphologically, both groups were very similar. The dendritic morphology of tyrosine hydroxylase and GABA immunoreactive processes was characterized by a darkly stained cytoplasm with several intracytoplasmic organelles and flattened vesicles dispersed throughout the cytoplasm. Both immunoreactive processes received type I, asymmetrical synapses from olfactory nerve terminals and mitral/tufted cell dendritic processes. The GABA and tyrosine hydroxylase processes made morphologically equivalent type II synapses characterized by pleomorphic vesicles juxtaposed to a symmetrical postsynaptic membrane specialization on identified mitral/tufted cell dendritic processes. No evidence was found where two immunoreactive processes synapsed onto one another or where an immunoreactive process received a type II synapse.

The major finding in this study was the synaptic distribution between these two groups. In both sets of immuno-processed material the mitral/tufted cell processes received the majority of olfactory nerve terminals. Although the immunoreactive tissues were not analyzed simultaneously, by using the non-immunoreactive periglomerular cells in a proportional comparison, one can deduce differences between the GABA and tyrosine hydroxylase immunoreactive periglomerular cells. Thus, the tyrosine hydroxylase immunoreactive cells received a higher number of synapses from olfactory nerve terminals compared to the GABA group. Mitral/tufted cell dendritic processes established a higher number of asymmetrical synapses onto the GABA group compared to the tyrosine hydroxylase. Finally, the tyrosine hydroxylase processes established a larger number of symmetrical synapses onto mitral/tufted cell processes than did GABA immunoreactive processes. These observations raise many questions especially in light





of the observation in Study I that the majority of periglomerular cells colocalize both GABA and tyrosine hydroxylase. These questions and potential answers will be discussed below.



## DISCUSSION

The intention of this thesis was to provide a better understanding of the synaptic organization of local circuits in the rat olfactory bulb glomerulus. Taking advantage of the anatomical and functional heterogeneity of the glomerulus, in three studies employing immunocytochemistry, light microscopy, and electron microscopy, the distribution and synaptic organization of periglomerular cells immunoreactive for GABA and tyrosine hydroxylase was assessed. The glomerulus serves as an attractive model for studying mechanisms of local circuit organization since it provides three elements that comprise the fundamental organization of local circuits; an afferent input (olfactory nerve), intrinsic neurons (periglomerular cells), and projection neurons (mitral/tufted cells). Thus, conclusions derived from the olfactory bulb glomerulus can be readily applicable to other areas of the central nervous system that involve local circuits.

### A. LIGHT MICROSCOPY

The antibodies used in these studies were against a neurotransmitter (GABA) and an enzyme, tyrosine hydroxylase. Nevertheless, tyrosine hydroxylase immunoreactivity is interpreted as a reliable indicator of dopamine synthesis in the rat olfactory bulb neurons. Tyrosine hydroxylase immunoreactive cells in the bulb do not immunoreact for dopamine- $\beta$ -hydroxylase or phenylethanolamine-N-methyl, which synthesize norepinephrine and epinephrine respectively (Halász et al., 1977). Moreover, other studies have reported an equivalent distribution of neurons employing antibodies directed against dopamine as well as different antibodies to tyrosine hydroxylase (Halász et al., 1977; Baker et al., 1983). Consequently, neurons immunoreactive to tyrosine hydroxylase are interpreted as dopaminergic neurons. The glomerular distributions of GABA and tyrosine hydroxylase immunoreactive cell bodies were consistent with previous observations (Halász et al., 1977; Baker et al., 1983; Davis and Macrides, 1983;



Kosaka et al., 1985; Gall et al., 1987). The vast majority of immunoreactive cell bodies in the glomerular layer were those identified as periglomerular cells.

The principal observations regarding periglomerular cells were in agreement with those described by Gall et al. (1987). In our study, the majority of immunoreactive cells colocalized GABA and tyrosine hydroxylase (71.8% compared to 69% in Gall et al. study). Approximately 26.6% of periglomerular cells were immunoreactive to GABA only (compared to 27% in Gall et al. study). In our study there was a small difference compared to Gall et al. in the number of periglomerular cell immunoreactive for only tyrosine hydroxylase. In our study 1.6% of periglomerular cells were immunoreactive for tyrosine hydroxylase only compared to 4% in their study. Another difference in observations involved the number of tufted cells immunoreactive to both GABA and tyrosine hydroxylase. In our study the majority of superficial tufted cells immunoreact for both GABA and tyrosine hydroxylase (81.9%) but there was a higher proportion of superficial tufted cells immunoreactive for tyrosine hydroxylase only (18.1%) compared to periglomerular cell (1.6%). Gall et al. reported the majority of tufted cells immunoreact for tyrosine hydroxylase only (93%) and only 7% colocalized both dopamine and GABA. Our study is in accord with Gall et al. in that there was no instance where a superficial tufted cell was immunoreactive for GABA only. The slight discrepancies between these studies might lie in technical factors such as differences in tissue penetration by different antibodies. The fundamental observations are consistent with the notion that there are at least three subpopulation of periglomerular cells. One that colocalizes both GABA and dopamine and two others that contain GABA or dopamine exclusively.

Not all cells in a glomerulus are immunoreactive to GABA or tyrosine hydroxylase. It was estimated that the number of periglomerular cells that contain GABA or dopamine comprised a small portion surrounding a given glomerulus. One of the drawbacks in estimating the total number of cell surrounding the glomeruli was that





the tissues sections were different from the ones that underwent the immunocytochemical process. Also, the cells counted in the 10 $\mu$ m thick sections included glial, short axon, periglomerular and superficial tufted cells. Therefore, the counts were not an absolute estimate of the number of periglomerular cells but reflected an approximation. A better study would have included 10 $\mu$ m sections, processed through an immunocytochemical-peroxidase reaction, and a cell count of immunoreactive process. The same sections could then be stained with cresyl violet and total periglomerular cell counts established. Nevertheless, it is clearly evident from the current study that there are many more periglomerular cells than have been accounted for by assessing dopamine or GABA immunoreactivity. Understanding the role of these cells in the glomerular circuits will be an important goal of future studies.

The biochemical diversity in the periglomerular cells could imply that, based on their neurotransmitters, these cells can be divided into functional categories. The colocalization of two classical neurotransmitters is seen rarely but has been encountered in neuromuscular junctions (Chan-Palay and Palay, 1982), raphe nuclei (Belin et al., 1983), arcuate nucleus (Everitt et al., 1984), and in the retina (O'Malley and Masland, 1989). It has been suggested that the periglomerular cells are responsible for lateral inhibition in the olfactory bulb. Converging lines of evidence suggest that the inhibition is mediated by GABA and to a certain extent by dopamine (Getchell and Shepherd, 1975; Nowycky et al., 1983). It is enticing to speculate that the presence of both neurotransmitters in the same cell can have a synergistic action on the mitral and tufted cells. However the differences in effect and mechanisms of action suggest that dopamine and GABA may have different postsynaptic targets and physiological actions. In the rat substantia nigra GABA has been shown to reduce the release of dopamine in the nigro-striatal fibers by activating GABA<sub>B</sub> receptors (Engberg et al., 1993; Engberg and Nissbrandt, 1993). Although periglomerular cells were not seen receiving a symmetrical inhibitory synapses (see above in Study II section), the presence of GABA<sub>B</sub> receptors in



the glomerular layer (Bowery et al., 1987) suggests the notion that perhaps in addition to inhibiting mitral/tufted cells, GABA also controls the release of dopamine. The action of GABA in the absence of synapses onto periglomerular cells could be mediated by GABA<sub>B</sub> autoreceptors in presynaptic periglomerular cells colocalizing both GABA and dopamine. This feedback loop could also inhibit periglomerular cells once these are excited by either olfactory nerve or mitral/tufted cells. The presence of GABA<sub>B</sub> autoreceptors in periglomerular cells remains to be established

Different physiological situations might arise where the action of dopamine or GABA would dominate. The release of GABA and dopamine by activation of periglomerular cells could produce a brief inhibition of mitral/tufted cells similar to that exhibited by GABA in other systems. However, prolonged stimulation of periglomerular cells may result in hyperpolarization of mitral/tufted cells in response to the summed effects of repeated dopamine exposure. Several studies have reported that the olfactory nerve adapts to prolonged odor stimulation, perhaps mediated presynaptically (Freeman, 1974; Mair, 1982). Moreover these adaptive changes occur in the presence of the GABA antagonist, picrotoxin.

The apparent intimate physiological relationship between dopamine and the olfactory nerve is further supported by the transsynaptic regulation of dopamine in periglomerular cells as seen following olfactory nerve transection and subsequent reinnervation of the bulb (Margolis et al., 1974; Keller and Margolis, 1975; Nadi et al., 1981; Baker, 1983). The transsynaptic regulation of dopamine may depend, in part, on trophic actions of the olfactory nerve on their periglomerular cell targets. In a different vein, dopaminergic D2 receptors have been identified on olfactory nerve synaptic terminals (Nickell et al. 1991). Thus the prolonged stimulation of dopaminergic periglomerular cells could result in activation of D2 receptors and an ensuing decreased release of excitatory neurotransmitters from the olfactory nerve terminals (Nickell et al., 1991). Assuming that a strong odor results in a prolonged and exaggerated excitation,



(Nickell et al., 1991), this negative feedback would allow the olfactory system to respond to a wider variety of odors in the presence of a strong odor.

If the action of dopamine was strictly that of controlling olfactory nerve activation, then this could help explain the small number of periglomerular cells immunoreactive to only tyrosine hydroxylase. Assuming that olfactory nerve axons are organized into odor specific fascicles and that they terminate in odor specific domains in the glomerulus, then a small number of dopaminergic periglomerular cells are required to control the actions of these terminals. The observation that only a small number of dopaminergic periglomerular cells control odor specific groups of olfactory nerve axons, support the notion of a functional columnar organization in the olfactory bulb suggested by other studies (Oron et al., 1984; Greer and Halász, 1987; Mori, 1987; Greer, 1991). Colocalization of GABA in these dopaminergic periglomerular cells would create a circuit where the release of dopamine is intimately linked to that of GABA, thus controlling the excitation of the olfactory nerve and creating a larger receptive field for a wider range of odors. Another potential role for dopamine is that similar to dopaminergic amacrine cells (Rogawski, 1987) where by uncoupling gap junctions, the receptive fields of the olfactory bulb are altered. GABA, on the other hand is required for inhibition of numerous mitral/tufted cell processes therefore requiring a larger number of periglomerular cells.

## **B. ELECTRON MICROSCOPY**

The purpose of the second study was to identify specific patterns of synaptic organization within the glomerulus employing electron microscopy and performing a systematic evaluation by location in the glomerulus. Assuming that a glomerulus is a sphere, our qualitative observations suggest the notion that there are differences in the type and density of synapses across individual glomeruli. A higher number of axodendritic synapses between the olfactory nerve and dendrites from periglomerular and





mitral/tufted cells was seen predominantly in the shell of glomeruli. In contrast, longitudinally oriented olfactory nerve axons were seen in the core, of glomeruli often without establishing an axodendritic synapse. These observations are in accord with 3-dimensional reconstructions suggesting that the olfactory nerve arborizes predominantly in the shell of the glomerulus (Halász and Greer, 1993). Although qualitatively it was difficult to assess any differences, a synaptological analysis shed some insights into the differential distribution of type I and type II dendrodendritic synapses across glomeruli.

Olfactory nerve terminals were always seen in islets and clustered together. There was never an instance where an individual olfactory nerve terminal was isolated from an islet. Similarly, dendritic processes from mitral/tufted and periglomerular cells were found in islets surrounded by olfactory nerve terminals. The dendritic terminals had synaptic and morphological characteristics that easily distinguished them from each other. Mitral/tufted dendrites for the most part had a regular shape and a light cytoplasm as compared to periglomerular dendrites which were irregularly shaped and contained a darker cytoplasm. Mitral/tufted cell dendritic processes were seen making type I asymmetrical synapses compared to periglomerular cell dendritic processes which made type II, symmetrical synapses.

The morphological observations of the glomeruli in this study are in accord with those previously described by Pinching and Powell (1971a,b) and White (1972, 1973). In their study, Pinching and Powell (1972b) observed the presence of reciprocal synapses between mitral/tufted cell and periglomerular cell dendritic processes more frequently than in our observations. This could possibly reflect the manner in which they performed their study. By employing serial reconstructions Pinching and Powell had an advantage of examining individual profiles for considerable lengths, thus providing a 3-dimensional view. Since reciprocal synapses are usually adjacent to each other, in a 3-dimensional study there would be a higher incidence of these synapses compared to our 2-dimensional assessment. A criticism of this study could lie in the manner in which the montages were



taken and if, in fact, they portray accurately the neuropil and synaptic components of the shell and the core of a given glomerulus. In this study a 3-dimensional spherical glomerulus was reduced to a 2-dimensional structure. It is plausible that some of the transglomerular montages in fact represented the shell more than the core if the montages were taken in the outer layer of the sphere. For this reason only large, round glomeruli that would represent as precisely as possible their components in the shell and the core were selected for the montages. An improvement on this study would include serial reconstructions of each probe in order to fully characterize the synaptology of individual dendrites. Needless to say this proposed study would require an enormous amount of time, supplies, and effort; in our case the inconvenience of gaining synaptological information from a 2-dimensional probe outweighed the more comprehensive approach of a 3-dimensional study.

Despite the potential shortcomings, this study established a foundation for the next study which employed immunocytochemistry to understand the synaptic distribution of dopaminergic and GABAergic periglomerular cells. This study also provided further evidence suggesting the glomeruli can be organized into subcompartments based on morphological and synaptological criteria. A subglomerular organization suggests that these compartments may subserve the formation of functional modules for odor processing. Similar observations of sensory system subcompartmentalization have been described in the whisker cortical barrel fields (Woolsey and Van der Loos, 1970), striate cortex (Lund, 1988) and retina (Dowling, 1987).

The third study combined the observations of the two previous studies in an attempt to characterize the synaptic organization of periglomerular cells containing GABA and dopamine. This is the first time that the differential distribution of synapses between subpopulations of intrinsic neurons in the olfactory bulb has been studied. Morphologically, both dopaminergic and GABAergic periglomerular cells were equivalent. They were irregular in shape, contained many flattened vesicles throughout

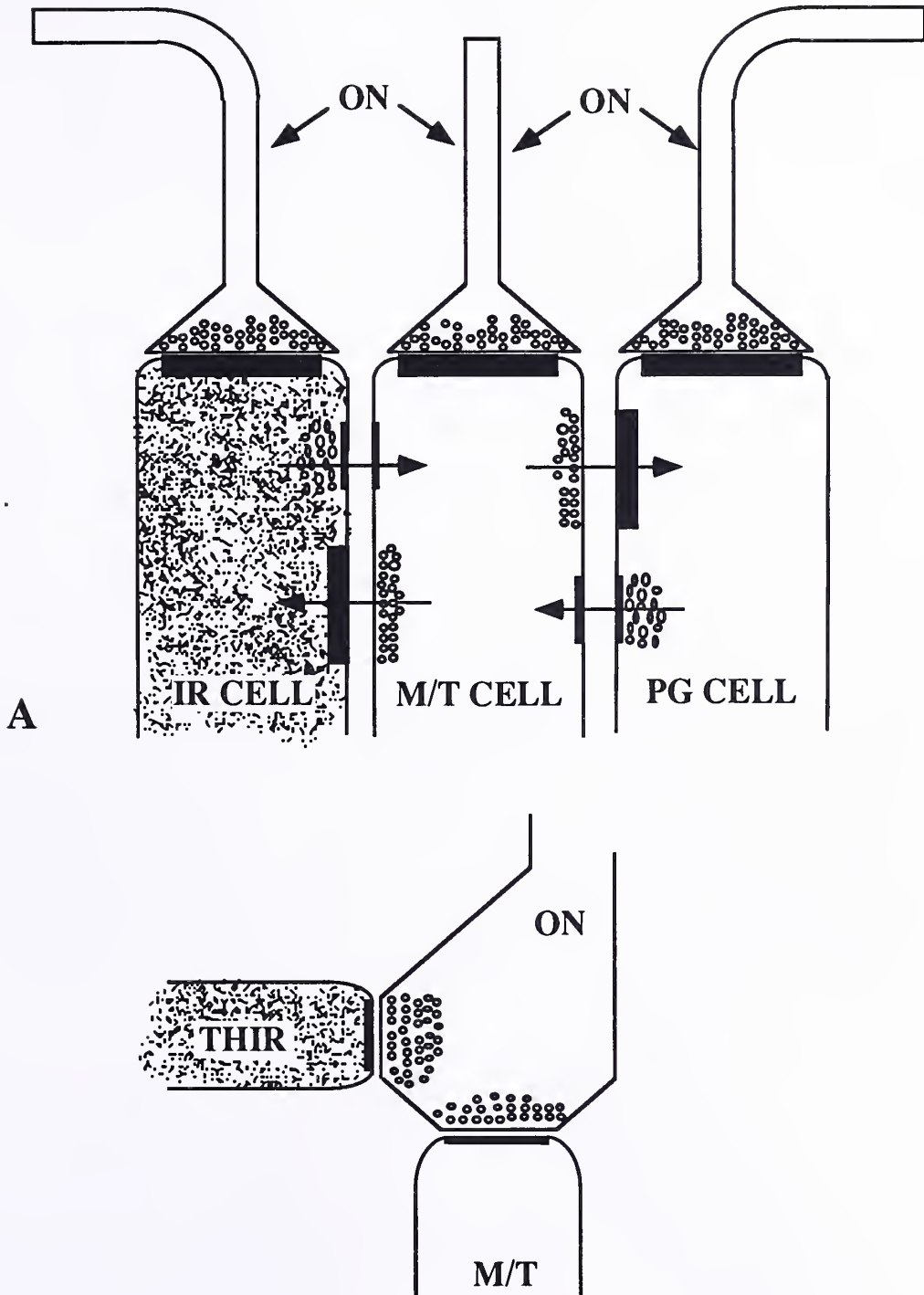


their cytoplasm and received and made equivalent synapses. Figure 56A summarizes the synaptic organization of immunoreactive and non-immunoreactive periglomerular cells in the olfactory glomerulus. Mitral/tufted cells and immunoreactive and non-immunoreactive periglomerular cells receive asymmetrical excitatory synapses from olfactory nerve terminals. The mitral/tufted cell dendrites make asymmetrical excitatory synapses characterized by spherical vesicles juxtaposed to an asymmetrical postsynaptic membrane specialization. The periglomerular cell dendrites in turn make symmetrical excitatory synapses onto mitral/tufted cell dendrites. These are characterized by pleomorphic vesicles juxtaposed to a symmetrical postsynaptic membrane specialization. In a few cases evidence of reciprocal synapses were observed but in the majority of cases symmetrical synapses were not adjacent to asymmetrical synapses and vice versa. There was only one instance in the tyrosine hydroxylase study (Figure 56B) where an olfactory nerve terminal made simultaneous synapses onto both a presumed mitral/tufted dendritic process and a tyrosine hydroxylase immunoreactive dendritic process. This observation raises the notion of divergence of information from a single nerve terminal. No other supporting example of divergence of information was seen in either the tyrosine hydroxylase or the GABA studies.

The major finding in this study was the differential distribution of synapses among mitral/tufted cells, non-dopaminergic/GABAergic periglomerular cells and both, dopaminergic and GABAergic periglomerular cells. Figure 57 illustrates the synaptic distribution in the glomerulus where arrows indicate proportional number of synapses per terminals. Mitral/tufted cell dendrites received a higher density of synapses from the olfactory nerve terminals. Among periglomerular cells dopaminergic cells received a higher density of synapses from olfactory nerve terminals compared to the other subpopulations of cells studied. In turn GABAergic periglomerular cells received a higher density of synapses from mitral/tufted cell dendrites compared to dopaminergic cells. Dopaminergic and GABAergic cell processes make symmetrical synapses onto







**B**

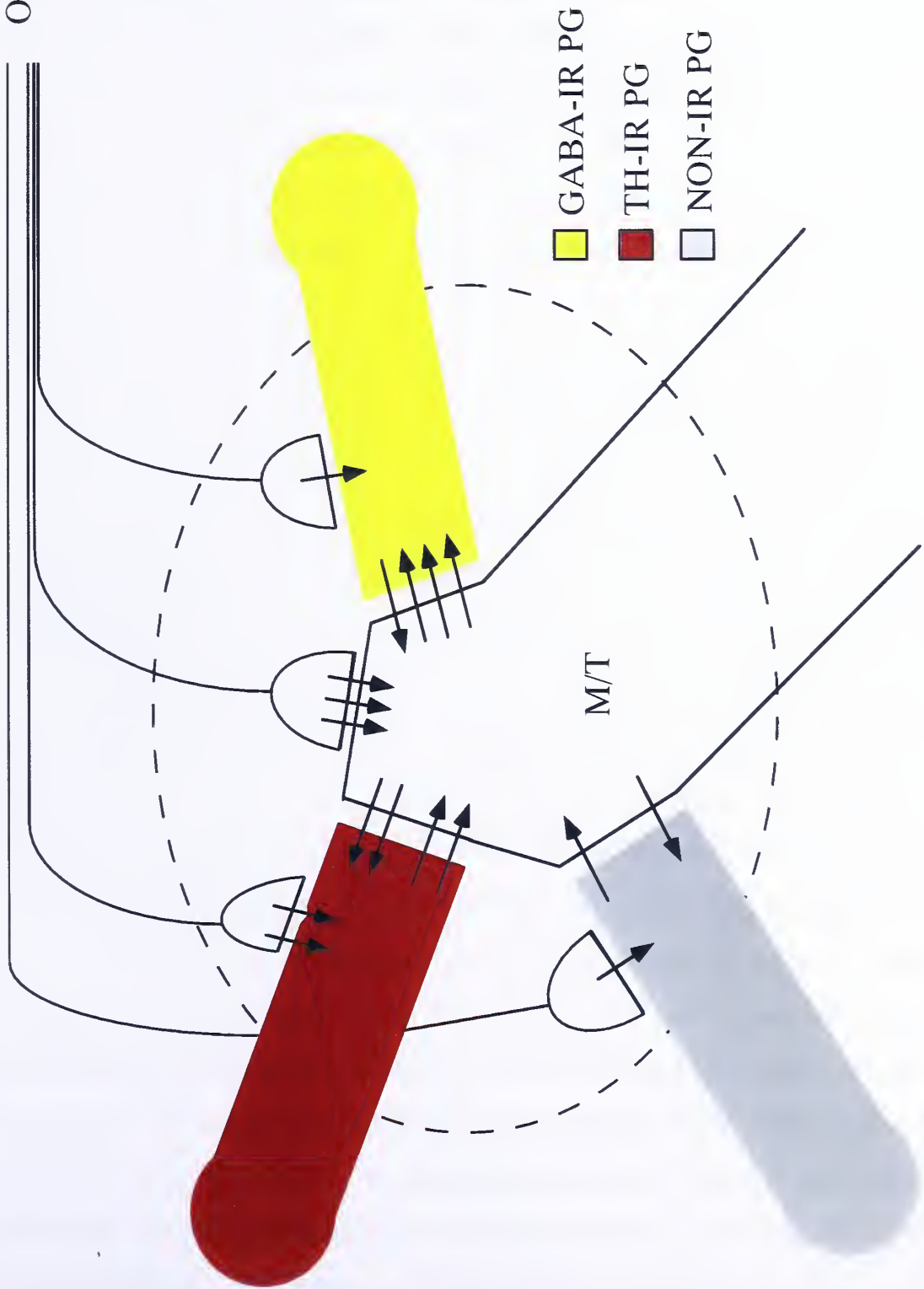
Figure 56. (A) Synaptic organization of immunoreactive processes for GABA and tyrosine hydroxylase. Immunoreactive processes (IR) receive asymmetrical synapses from olfactory nerve terminals (ON) and mitral/tufted (M/T) cell processes. Immunoreactive processes make symmetrical synapses onto mitral/tufted processes. (B) Example of divergence of information. Olfactory nerve makes simultaneous synapses onto a tyrosine hydroxylase immunoreactive process and mitral/tufted cell process.





Figure 57. Synptological characterization of GABA and tyrosine hydroxylase immunoreactive, non-immunoreactive and mitral/tufted cell dendritic processes. Arrows indicate polarity and proportional number of synapses that processes receive. Notice that mitral/tufted cell processes receive a greater number of synapses from olfactory nerve terminals. Tyrosine hydroxylase immunoreactive processes receive a greater number of synapses from olfactory nerve terminals than GABA immunoreactive processes. On the other hand, GABA immunoreactive processes make a greater number of synapses onto mitral/tufted cell processes. Abbreviations: GABA IR, GABA immunoreactive dendritic processes; TH IR, tyrosine hydroxylase immunoreactive processes; PG, periglomerular cells; M/T, mitral/tufted cell processes; ON, olfactory nerve.

ON







likely mitral/tufted cell processes, although the density is somewhat higher for dopaminergic than for GABAergic processes. The non-immunoreactive periglomerular cells had an intermediate density compared to the tyrosine hydroxylase and GABA groups. The synaptic distribution observed in this study certainly supports proposed physiological implications discussed at the beginning of this section. The fact that olfactory nerve terminals preferentially make synapses onto dopaminergic periglomerular cells supports the notion that dopamine could be responsible for olfactory nerve modulation through D2 receptors, despite the fact that no conventional synapses were seen onto olfactory nerve terminals. Also, this differential distribution could explain why olfactory nerve deafferentation results in decreased expression of dopamine in periglomerular cells. The transsynaptic control of dopamine expression is linked to the density of synapses from olfactory nerve terminals. Once these nerve terminals are removed then there is no need for the modulation of these terminals, thus dopamine expression is decreased. As the olfactory nerve regenerates and reinnervates the bulb, a physiological situation arises where a necessary mechanism to attenuate olfactory nerve transmission is needed. This results in increased expression of dopamine. The preferential distribution of synapses from mitral/tufted dendrites onto GABAergic neurons suggests that the primary role of GABA is that of inhibiting mitral/tufted cells.

If the majority of periglomerular cells studied colocalized both GABA and dopamine and morphologically these were equivalent then why is there such a difference in their synaptic distribution? A plausible answer to this might lie in how the study was performed. We were unable to study the three subpopulation of periglomerular cells at the same time. We studied the dopaminergic separate from the GABAergic cells therefore we were not able to simultaneously characterize those that colocalized both neurotransmitters. Therefore, our results express the synaptic distribution of three subpopulations of periglomerular cells. In an attempt to compensate for this potential



bias and make micrographs as equivalent as possible, we were very careful in trying to include in every micrograph neuronal elements such as olfactory nerve terminals, dendritic processes and synapses. Perhaps during our sampling we studied those cells that expressed only one neurotransmitter. That is, in the GABA study only the 26.6% of periglomerular cells that contain GABA only were sampled and in the tyrosine hydroxylase group we sampled a combination of colocalizing and dopaminergic only periglomerular cells. However, based on the large percentage of cells colocalizing both neurotransmitters and on our sampling of many neuronal processes, this scenario seems highly unlikely. Nevertheless, we could improve this study making serial reconstructions, employing postembedding immunogold labeling and using different size gold particles covalently linked to antiserum against GABA and tyrosine hydroxylase. This technique would allow the clear visualization with a 3-dimensional perspective of dendritic profiles labeled for each of the neurotransmitters of interest. Furthermore, colocalizing periglomerular cell dendritic processes would be easily identified as containing both sizes of gold particles, thus allowing the investigator to study and characterize simultaneously the three subpopulation of periglomerular cells.

It is evident from our results that there exists a heterogeneous synaptic distribution from olfactory nerve terminals onto subpopulations of intrinsic and projection neurons. Mitral/tufted cells also express a heterogeneous synaptic distribution onto subpopulations of periglomerular cells. The differential synaptic distribution between GABAergic and dopaminergic periglomerular cells onto mitral/tufted cells could be the result of non-classical synaptic contacts. Dopaminergic cells in the striatum are not closely associated with postsynaptic specialization (Tennyson et al., 1974). Similarly, Schwartz (1987) has reported a carrier mediated, non-vesicular calcium independent mechanism for GABA release in amacrine cells of the retina. However, another study reported GABAergic amacrine cells making conventional synapses in the cat retina (Chun and Wässle, 1989). Amacrine cells are known to colocalize more than



one neurotransmitter/neuromodulator and perhaps these conventional synapses were from other neurotransmitter/neuromodulator. In a similar vein, the synapses from immunoreactive processes reported in our study could be those of other neurotransmitters/neuromodulator supporting the observation of a differential synaptic distribution. It could be deduced that the difference in synaptic connectivity exemplifies different modulatory roles and postsynaptic targets for GABA and dopamine in the glomerular layer of the olfactory bulb. Additional anatomical and physiological studies are needed to fully characterize the neurochemical properties of periglomerular cells and their functional role in odor processing.

In summary, utilizing the olfactory bulb as an exemplary model for studying local circuits, this study attempted to describe mechanisms of synaptic specificity and organization in the rat olfactory glomerulus. In the first study the heterogeneous distribution of dopaminergic and GABAergic periglomerular cells was assessed under the light microscope. These observations served as a launching pad for the following two studies, which attempted to fully characterize the synaptological arrangement between subpopulations of periglomerular cells, mitral/tufted cells and olfactory nerve terminals. The observations of a differential synaptic distribution found among the neuronal elements in the glomerulus supports the notion of an organizational heterogeneity within the glomerular structure.

### **C. FUTURE DIRECTIONS**

Ongoing studies in the laboratory are attempting to elucidate mechanisms of axonal guidance and defasciculation from the olfactory epithelium into the bulb by assessing the distribution of cell adhesion molecules within olfactory nerve fibers. The expression of these molecules has been shown to vary between glomeruli (Miragall et al., 1989; Key and Akeson, 1990; Miragall et al., 1990). It is hypothesized that proximal to the olfactory epithelium there is a homogenous expression and distribution of cell





adhesion molecules in the olfactory nerve layer. As the olfactory nerve reaches the glomerular layer, the expression of these molecules is altered allowing defasciculation and penetration of these fibers into individual glomeruli.

Studies from different laboratories have shown that fascicles within the olfactory nerve layer and glomeruli exhibit molecular specificity through the expression of lectin binding or a variety of cell surface glycoconjugates (Mori, 1993). Recently Buck and Axel (1991) reported that individual olfactory receptor cell axons expressed molecular specificities for potential receptor proteins, suggesting the notion that individual receptor cells are responsible for individual odors. Combining these two observations and using antibodies against these molecular markers it is plausible to study the trajectory of fascicles or individual olfactory nerves and their synaptic organization within individual glomeruli. An interesting new study could employ postembedding immunocytochemical double labeling of periglomerular cell and olfactory nerve terminals with gold particles of different sizes. The anatomical and functional relationship between olfactory nerve and, for example, dopaminergic periglomerular cells can be corroborated, thus enhancing our understanding of synaptic specificity.

Understanding the specific patterns of innervation of olfactory nerves could further support the notion of subglomerular organization subserving functional modules for odor recognition. Also, the continual regeneration expressed by the olfactory system allows for a better understanding of fundamental molecular and genetic principles governing axonal guidance and synaptic specificity. These principles can potentially be applicable in neuronal transplants as a means to establishing synaptic and functional reorganization.

Finally it is the observation of the author that history involving the olfactory system seems to repeat itself. The trivial interest placed in the olfactory system by clinicians results from the limited clinical and interventional applications that the olfactory system offers. In a fashion similar as Golgi and Cajal, applying today's



molecular and genetic techniques the olfactory system could potentially elucidate revolutionary discoveries that would set the standards governing mechanisms of neuronal synaptic specificity, plasticity and regeneration. These discoveries could have a significant impact and broad application in clinical settings. As Thomas Lewis said (1983):

“... We might fairly gauge the future of biological science, centuries ahead by estimating the time it will take to reach a complete, comprehensive understanding of odor. It may not seem a profound enough problem to dominate all the life sciences, but it contains, piece by piece, all the mysteries.”



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